

**STUDIES ON THE ORAL DELIVERY OF PHOSPHOCITRATE**

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by

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Except as stated herein, this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and that, to the best of my knowledge and belief, this thesis contains no paraphrase of material previously published or written by another person, except where due reference is made in the text of the thesis.

*b. booper.*

Chris Cooper

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## ABSTRACT

Phosphocitrate is a potent inhibitor of hydroxyapatite formation with demonstrated efficacy in preventing the progression of calcific pathologies in animal models. In the present studies, the oral bioavailability of phosphocitrate was investigated following administration of the compound by gavage in rats. Using a calcergic model, inhibitory action by phosphocitrate was evaluated. Accordingly, the formation of small subcutaneous calcific plaques was chemically induced. Parameters measured included plaque weight, calcium, and phosphorus content. The oral and intraperitoneal routes of administration were compared and dose-response relationships established. Phosphocitrate was found capable of reducing calcific plaque formation when delivered by either route. However reduced bioavailability was apparent following administration by gavage revealing the oral route to be approximately 45 times less effective on a dose-weight basis than the intraperitoneal route.

The possibility that poor absorption from the small intestine was responsible for the observed differences was explored. Transport studies using everted sacs and *in situ* ligated loops confirmed phosphocitrate absorption. The data revealed concentration-dependance and the absence of *in vitro* intestinal transfer at the lowest concentration (1mM) trialled.

Further studies were necessary to evaluate the extent of phosphocitrate absorption from the small intestine. Experiments with radiolabelled phosphocitrate and using an *in situ* perfusion technique with hepatic portal vein cannulation yielded additional information. The studies provided conclusive evidence that limited amounts of intact phosphocitrate were being absorbed. First pass metabolism to citric acid was observed but this metabolism was not commensurate with the poor rate of absorption. Studies utilising tricarballlylate,

a known inhibitor of tricarboxylic acid transport in the intestine, indicated that the disappearance of phosphocitrate from the intestinal lumen was not affected by the presence of this transport inhibitor. Consideration of this and everted sac data gave credence to the suggestion that phosphocitrate transport did not involve an established tricarboxylic acid transporter.

A preliminary investigation was made into the possibility of improving the oral bioavailability of phosphocitrate with aliphatic ester prodrugs of phosphocitrate. Initially the approach was to investigate the synthesis and ultimate benefit of short (ethyl) and medium chain (octyl) esters. The triethyl ester of phosphocitrate was synthesised and trialled but the isolation and purification of the trioctyl ester was not possible within the time frame of these studies.

Conclusions reached in this study were that phosphocitrate was absorbed following oral administration but that the extent of absorption was limited. Triethyl phosphocitrate was prepared in an attempt to improve the transport characteristics of phosphocitrate. Plaque calcification was not reduced following the administration of this ester. The prodrug approach was discussed as a possible avenue for improving the absorption of phosphocitrate.



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## CHAPTER 1

### INTRODUCTION

#### 1.1 CALCIFICATION - CALCIFIC DISEASES

Calcification is a widespread biological phenomena occurring either as a physiological or a pathological process. It is the most ubiquitous of all the biomineralisation processes where two thirds of all biominerals contain calcium (Lowenstam, 1981). Calcium phosphates, specifically hydroxyapatite, are the most common types of calcium salts in higher vertebrates but additionally, carbonate, oxalate and pyrophosphate salts also occur (Russell *et al.*, 1986).

The regulation of normal calcification is a complex procedure involving the controlling activity of a range of effectors. These include collagen, pyrophosphate, proteoglycans, osteocalcin and osteonectin (Williams and Frolik, 1991) which are in turn inhibited or stimulated by a further range of hormones and growth factors. Two general theories are proposed for the mechanism of initiation of calcification (Williams and Frolik, 1991). One of these, the nucleation theory, states that for calcification to occur a highly ordered nucleation site must exist. This site, which is believed to be created by collagen fibrils, ensures an ordered interaction of crystal substrates in a spatial conformation which allows crystallisation to occur. The second theory focuses on the involvement of matrix vesicles in the initiation process whereby these vesicles have various functions including, concentrating calcium and phosphate at the nucleation site and providing enzymes for the removal of inhibitors to crystal growth. However it is possible that both theories have a role, such that in mature bone, calcification is initiated by nucleation whereas in cartilage and

early embryonic bone, where there is no obvious relationship between collagen and initial crystallisation, the process is controlled by matrix vesicles.

The functional necessity of normal calcification in bone and teeth formation is obvious but unfortunately abnormalities, including osteoporosis (Nordin *et al.*, 1984) and Paget's disease (Russell, 1984), arise when lack of control occurs. Additionally, a vast range of pathologies have been described which involve the abnormal calcification of tissues other than bone (Table 1.1) and these are usually referred to as ectopic calcification (Anderson, 1983). Sometimes a characteristic bone matrix forms (termed heterotopic calcification), and includes diseases such as myositis ossificans progressiva, post-surgical or trauma complications and ankylosing spondylitis (Russell and Kanis, 1984).

Calcification which does not involve the formation of an identifiable bone matrix is traditionally subdivided into two groups, namely dystrophic and metastatic calcification. Intrinsic in the mechanism of metastatic calcification is the elevation of circulating levels of calcium and phosphate. Resultant alterations in the physicochemical and physiological equilibria can result in the formation of amorphous calcium phosphate leading to crystal formation. Metastatic calcification is common in the kidney, stomach and vascular system and has been associated with hyperparathyroidism, neoplasms, milk-alkali syndrome, hypervitaminosis D and tumoral calcinosis (Black and Kanat, 1985).

Dystrophic calcification however does not involve elevated serum calcium and phosphate levels but is a response to tissue injury. The mechanism of initiation of this form of calcification is poorly understood but the

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**Table 1.1 Summary of Calcification Types**

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**Normal Calcification**

Bone, teeth and cartilage

**Pathological Calcification**

*Heterotopic*

Involves a characteristic bone matrix

- myositis ossificans progressiva
- post surgical and trauma complications
- paraspinal calcification (e.g. ankylosing spondylitis)

*Metastatic*

Calcification results from hypercalcaemia or hyperphosphataemia

- tumoral calcinosis
- hypervitaminosis D
- neoplasms
- milk alkali syndrome
- hyper parathyroidism
- chronic renal failure

*Dystrophic*

A response to tissue injury (e.g. necrosis, trauma)

- monckebergs medial calcinosis
- scleroderma
- tympanosclerosis

*Crystal deposition disorders*

- hydroxyapatite
- octacalcium phosphate
- tricalciumphosphate
- calcium pyrophosphate

*Urolithiasis*

Calcium oxalate and/or calcium phosphate stones

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(based on Russell and Kanis, 1984)

involvement of mitochondria and/or matrix vesicles in the accumulation of calcium following damage to homeostatic mechanisms is almost certainly involved (Anderson, 1983). Diseases in which dystrophic calcification is observed include scleroderma, dermatomyositis and Monckebergs medial calcinosis. Further, the process can be a natural part of ageing especially as regards calcification of the pineal gland and blood vessels.

Several widespread, major disease states involve calcification as part of their progression. Atherosclerosis (McGill, 1988) is a major calcific disease involving dystrophic calcification. The term atherosclerosis actually refers to a number of disease states but all primarily involve anatomical and functional degeneration of some part of the vascular system. Fleckenstein *et al.* (1990) regard calcium overload of the arterial walls as a primary pathogenic factor leading to arterial destruction. Additionally, atherogenesis involves calcium mediated stages including changes in membrane permeability, cell proliferation, cell migration and the secretion of extracellular matrix proteins (Kjeldsen and Stender, 1989).

Other common disease states which are broadly associated with calcification are those involving crystal deposition in joints. These diseases are varied and involve the appearance in and around joints of calcium salts. Basic calcium phosphate (BCP) deposition encompasses the appearance of octacalcium phosphate, hydroxyapatite or tricalcium phosphate (whitlockite) in joints (Halverson and McCarty, 1989). In addition calcium pyrophosphate dihydrate (CPPD) (Ryan and McCarty, 1989) and calcium oxalate (Reginato, 1989) deposition have also been described. The appearance of crystals in joints can lead to the destruction of surrounding membranes and may result in severe ongoing trauma and inflammation (Terkeltaub *et al.*, 1989).

Urolithiasis is a broad term which refers to the precipitation of insoluble components of urine leading to the deposition of crystals (calculi) within the urinary tract (Robertson, 1984). It is estimated that between 50 and 80% of stones contain calcium, mainly present as calcium oxalate and sometimes as calcium phosphate. Of the remainder, urate, magnesium ammonium phosphate and cysteine stones are frequently observed. However it is thought that small crystals composed of calcium phosphate can provide a nidus for the growth of most stones.

Modern surgical techniques have created their own problems with regard to calcification. Encrustation of implants and catheters with calcific deposits can affect their longevity. Calcium phosphates have been detected in mineral deposits attached to urinary catheters (Cox and Hukins, 1989) and Levy *et al.* (1985) has described similar encrustations associated with bioprosthetic heart valve implants. Dysfunction in the later case can result from stenosis or secondary calcific tearing of the leaflets (Johnston *et al.*, 1990).

## 1.2 INHIBITION OF CALCIFICATION

Given the myriad types of calcific diseases, it is clear that effective inhibitors might be seen as crucial in preventing the initiation or progression of these disease processes. The metastable nature of calcium and phosphate ions in extracellular fluids has long suggested the possibility that naturally occurring inhibitors of calcium salt formation might exist. Sindhu *et al.* (1989) listed magnesium, zinc, fluoride, stannous ion, citrate, pyrophosphate, ribonucleic acid, and acidic glycoproteins as naturally occurring inhibitors of urolithiasis. In fact urine itself has a noticeable inhibitory effect on hydroxyapatite crystal

formation (Hansen *et al.*, 1976) and Ogawa *et al.* (1986) found that salts of pyruvic acid and bicarbonate inhibited experimental oxalate urolithiasis.

Meyer and Fleisch (1984), whilst suggesting that quantitatively most of the inhibitory power responsible for metastability in blood was probably due to high molecular weight substances, recognized that magnesium, pyrophosphate and citrate ions are important low molecular weight inhibitors of calcification in blood. Naturally occurring pyrophosphate and longer chain synthetic polyphosphates are powerful inhibitors of calcification *in vitro* (Francis, 1969). However these compounds were found to be highly unstable *in vivo* (Russell and Smith, 1973). Jung *et al.*, (1970) found that pyrophosphate disappears rapidly from the bloodstream. Additionally, the compound is not effective when administered orally (Fleisch and Russell, 1977). These overall characteristics leave pyrophosphate unsuitable for clinical use.

The search for more stable analogs of pyrophosphate led to the examination of a group of compounds now known as the bisphosphonates (diphosphonates). The P-O-P bonding arrangement of pyrophosphate is replaced by a P-C-P arrangement in the diphosphonates adding stability but unfortunately rendering the molecules enzymatically non-metabolisable. These compounds have been trialled extensively and shown to be very effective inhibitors of ectopic calcification (reviewed by Russell and Smith, 1973; Fleisch and Russell, 1977; Fleisch, 1981; Fleisch, 1983) and unlike pyrophosphate the diphosphonates can be absorbed from the intestine (Michael *et al.*, 1972), although the amounts transported are usually very low.

Etidronate (EHDP), the most commonly reported diphosphonate has been shown to inhibit vitamin D3 induced aortic calcification in rats (Francis *et*

*al.*, 1969). However complications in the long-term administration of diphosphonates have limited their use. The diphosphonates have cellular effects and possible toxic implications in terms of bone growth (Miller *et al.*, 1985). Dichloromethylidene biphosphonate ( $\text{Cl}_2\text{MDP}$ ), another compound in the series, which has been used to treat malignancy induced hypercalcaemia has nephrotoxic potential (Alden, 1990). Overall, the toxicity and poor oral absorption of diphosphonates has resulted in these compounds having limited clinical use. However  $\text{Cl}_2\text{MDP}$  and 4-amino-1-hydroxybutylidene-1,1-diphosphonate, unlike EHDP, have the advantage of not inhibiting skeletal mineralisation at doses that are required for inhibition of bone resorption and are used in the treatment of Paget's disease (Adami *et al.*, 1986)

A totally different class of compounds which may exert strong influence on calcific processes are the calcium antagonists (Godfraind, 1987). These agents modulate the entry of calcium into cells through selective or nonselective mechanisms. The compounds have strong anti-hypertensive action and effects on the vascular system, heart, cardiovascular hormones and renal function have been observed (Struyker-Boudier *et al.*, 1990). They have been trialled as antiatherogenic agents in animal models and might conceivably prevent the toxic effects of excessive calcium accumulation by cells. The inhibition of arterial smooth muscle cell proliferation may also play a role (Struyker-Boudier *et al.*, 1990). However definitive conclusions concerning their usefulness as inhibitors of atherogenesis have not been made (Kjeldsen and Stender, 1989).

Concerning anticalcifying compounds generally, it is recognised that for a compound to have anticalcifying ability certain structural requirements exist (Williams and Sallis, 1982), as for many the mode of action of these compounds is thought to be via binding to charged sites on the growing crystal surface.



Termine and Conn (1976) suggested that the presence of two phosphate moieties within the inhibitor molecule was important. More recently Williams and Sallis (1982) conducted detailed studies which indicated that strong inhibition could be obtained with compounds which contained one phosphate and multiple carboxylic acid groups in close proximity.

### 1.3 PHOSPHOCITRATE - A PERSPECTIVE

Phosphocitrate (PC) (Figure 1.1) is one of the most potent naturally occurring anticalcifying agents so far recognised. The molecule has a highly polar nature and it is the spatial arrangement of the ionisable groups, whereby three carboxyl groups and a phosphate moiety radiate from a tetrahedral carbon atom, which appears to confer the powerful inhibitory potential (Williams and Sallis, 1979; Williams and Sallis, 1982). PC is naturally occurring being found in low concentrations in rabbit liver and kidney mitochondria (Williams and Sallis, 1981) and possibly the hepatopancreas of the blue crab (*Callinectes sapidus*) (Lehninger, 1983). In addition, synthetic PC is available as a result of synthetic strategies devised in three different laboratories (Meyer *et al.*, 1959; Williams and Sallis, 1980; Tew *et al.*, 1980).

Whilst it was first hinted by Meyer *et al.* (1959) that PC might have biological significance, the first real notions concerning the implications of PC as an anticalcifying agent came in a publication by Howard (1976). However until sufficient synthetic material became available in the early 1980's PC could not be tested. Using non-biological physical-chemical assays, PC has been found to be a powerful inhibitor of amorphous calcium phosphate conversion to hydroxyapatite (Williams and Sallis, 1979; Reddi *et al.*, 1980; Tew *et al.*, 1980; Williams and Sallis, 1982) and also to inhibit *in vitro* calcium oxalate formation

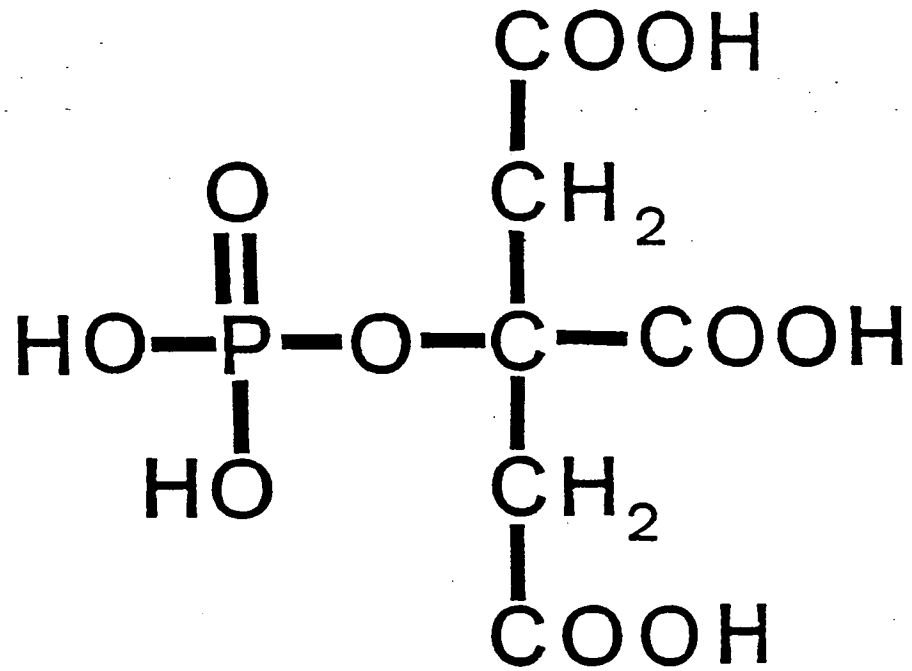


Figure 1.1 The structure of PC

(Williams and Sallis, 1981). Further, early *in vivo* studies investigating the role of PC as an inhibitor of mitochondrial calcification (Tew *et al.*, 1981a; Lehninger, 1983) and nephrocalcinosis (Tew *et al.*, 1981b; Gimenez *et al.*, 1982) confirmed that PC was a powerful anticalcifying agent *in vivo*.

Since then PC has been studied using various experimental animal models that describe calcific disease processes. Concerning urolithiasis, the inhibition of nephrocalcinosis by PC might imply that the inhibitor has potential to be active in the inhibition of calcium phosphate urinary stone formation. Certainly, it has been demonstrated that PC can reduce mixed magnesium (struvite and newberyite) stone formation (Sallis *et al.*, 1988). This data was interesting because it showed that PC can influence mineral formation outside of calcium containing structures. However, regarding calcium oxalate, the main type of calcium containing urinary stone, PC did not appear to have a strong influence *in vivo* (Brown and Sallis, 1985; Sallis *et al.*, 1989).

One area of potential usage for PC is in the field of crystal deposition disorders. The alleviation of hydroxyapatite and calcium pyrophosphate crystal induced polymorphonuclear leukocyte membranolysis by PC (Sallis *et al.*, 1989) alludes to the possibility that this compound may have an important role in ameliorating the damaging effects of osteoarthritis and chondrocalcinosis. More recently, Cheung *et al.* (1990) have demonstrated inhibition of basic calcium phosphate crystal induced mitogenesis by PC. Specifically PC inhibits crystal induced expression of *c-fos* in a way that is suggestive of PC acting at a cellular level.

Another field that has been explored with respect to the actions of PC is atherosclerosis (Shankar, 1986). Accumulation of calcium, by myocardial fibres,

vascular smooth muscle cells and elastic elements in the arterial walls, can be cytotoxic leading to the deposition of calcium salts (Fleckenstein *et al.*, 1990). The inhibition of dystrophic calcification of arteries by PC was demonstrated using an aortic transplant model (Shankar *et al.*, 1984b) and further to this PC has been reported to inhibit  $^{45}\text{Ca}^{2+}$  uptake by smooth muscle cells in primary culture (Shankar and Sallis, 1985). This latter finding is interesting because it appears that a direct cellular action could have occurred. Additionally it was observed that PC acted by a different mechanism to the calcium channel blocker diltiazem, because when the compounds were applied together their effects were additive. Consequently a possibly unique therapeutic role for PC in atherogenesis was proposed.

In experiments with a hyperlipidaemic rat model PC was found to reduce monocyte adhesion to arterial endothelium. This latter finding also has important implications that PC may be acting at a cellular level. Further to this PC inhibits low density lipoprotein (LDL) (Ward *et al.*, 1987) uptake and degradation by aortic smooth muscle cells. This process is thought to at least partly involve competition between PC and LDL receptors for binding to positive domains on the lipoprotein. However a direct cellular effect may be possible because heparin on a molar basis was less effective than PC. Interestingly, phosphoisocitrate which has the same charge as PC but a different chemical structure was less active in preventing LDL uptake. It is possible that PC, based on its high negative charge to size ratio and specific stereochemistry may have a range of possible *in vivo* effects that involve binding to regions of positive charge.

Following the indications that PC had anti-atherogenic potential, Tsao *et al.* (1988) investigated the compound with respect to its effect on the

calcification of bovine pericardium used in bioprosthetic heart valves. Using a rat model, dystrophic mineralisation of these valves was found to be decreased following local delivery of PC with subcutaneously implanted osmotic minipumps. The authors were unable to detect any adverse effects and concluded that PC may have future clinical importance in this area.

PC trials have so far been restricted to experimental animal models leaving therapeutic and any potential toxicity in humans to be determined. One of the problems with using anticalcifying agents at therapeutic dose levels is the potential for toxic effects on normal bone mineralisation. To date no evidence of toxicity has been found, as might be expected given that PC is found in soft tissue mitochondria and that the likely breakdown products of PC, citrate and phosphate, are also naturally occurring. Additionally, an enzymatic biological synthesis of PC has recently been reported (Moro *et al.*, 1990). More importantly Reddi *et al.* (1980) found an absence of effect on bone and cartilage mineralisation, and Tew *et al.* (1981b) found that PC did not have any detrimental effects on bone growth. Shankar (1986) conducted studies (up to 90 days) investigating metabolic parameters and bone histology, finding no side effects. Actually the compound was observed to normalise some adverse effects of an arteriosclerotic diet by normalising serum creatinine and uric acid levels.

#### **1.4 ORAL ABSORPTION**

The potential of PC to be used as a drug has not been fully delineated to date and one of the major questions to be answered concerns administration. Being mindful that clinically oral delivery is preferred, the most efficient way to administer PC needs to be determined. Drug efficacy itself depends on

absorption, distribution, biotransformation and elimination. These are dynamic processes which contribute to the bioavailability of a drug species and ultimately determine the usefulness of a drug. Many factors including psychological barriers preclude the use of some routes of administration for drugs leaving oral dosing as the preferred option. In this respect the question of PC oral availability is crucial and needs to be examined before the development of appropriate dosage forms.

Gastrointestinal absorption is complex, being influenced by a variety of physiological and physicochemical parameters. Kararli (1989) includes gastrointestinal motility, gastric emptying, intestinal blood and lymph flow, bile salts, surface pH of the mucosa, the unstirred water layer and colonic microflora as relevant factors. Absorptive mechanisms exist at different sites within the alimentary canal. Absorption of drug molecules from the stomach has been observed experimentally, but is not generally regarded as significant if gastric emptying is unimpeded. Buccal and colonic absorption while notable for some compounds will not be considered in detail here because intestinal absorption is the most common route of entry into the body for drugs and needs to be explored first. The mucosal barrier (Houston and Wood, 1980) is quite complex. The lumen contents are separated from the mucosal cells by a 300 micron unstirred water layer which maintains an acidic microenvironment extending 20 microns from the microvillus. The acidic microenvironment has important consequences with respect to ionization of charged groups. The microvillus and associated surface coat present the final barrier to transport.

Having reached the mucosal surface of an epithelial cell, a drug molecule has five pathways at its disposal for transport across the membrane. Schwenk (1987) describes the mechanisms of paracellular diffusion,

transcellular diffusion, facilitated diffusion, active transport and endocytosis in relation to solute absorption. Most drugs, unless they have a direct similarity to biological species which are normally absorbed via active transport mechanisms, are absorbed by the passive transcellular route (Houston and Wood, 1980). In addition, Schwenk (1987) regards the paracellular route as important for many compounds. Here the motive force for absorption is the solvent drag that accompanies the paracellular absorption of water.

Regarding the intestinal transport characteristics of PC there appears to be little information available in the literature which might help answer the question of PC absorption. The absorption of citrate and other tricarboxylic acid cycle intermediates from the gastro-intestinal tract has been poorly researched. Browne *et al.* (1978) studied citrate absorption from the small intestine of the hamster using everted sacs, and presented evidence that a specific mechanism is involved with absorption. A saturable Na-dependant mechanism was observed and furthermore, this transport was against a concentration gradient. Because tricarboxylic acid intermediates are extensively metabolised during transport (Browne *et al.*, 1978), Wolfram *et al.* (1990) used brush border membrane vesicles for their transport studies. The latter study reported a pH effect whereby the protonated species (citrate<sup>2-</sup> and citrate<sup>1-</sup>) were better transported than the trivalent species.

The possibility of PC transport being by the same mechanism as described for intestinal citrate uptake is not known. Certainly it would be anticipated that physicochemical parameters might determine the rate of PC absorption across the mucosal barrier. Factors, which include molecular size, lipophilicity and ionisation, that might influence absorption are discussed in

several general reviews and books (Houston and Wood, 1980; Peng, 1983; Kararli, 1989; Ansel and Popovich, 1990).

For PC the ionisability of the phosphate and carboxylate groups is especially relevant with respect to transcellular diffusion. For most water soluble drugs, absorption potential can be described according to the Henderson Hasselbach equation ( $\text{pH} = \text{pK}_a + \log \text{Ci}/\text{Cu}$  for acids, where  $\text{Ci}$  = ionised concentration and  $\text{Cu}$  = unionised concentration). Effectively this states that as the pH is lowered, for any given  $\text{pK}_a$ , the ratio of unionised to ionised compound is increased. A large body of evidence supports the concept that most drugs are absorbed in the unionised form (Schanker, 1971). Hence from a consideration of the  $\text{pK}_a$ s for PC (Ward, 1986), which are  $\text{pK}_{a1} = <2$ ,  $\text{pK}_{a2} = 3.67$ ,  $\text{pK}_{a3} = 5.15$ ,  $\text{pK}_{a4} = 7.69$ ,  $\text{pK}_{a5} = 13.56$ ; it is immediately apparent that PC will always be highly charged at the physiological pH of the intestinal lumen.

The ionisation constant data for PC allows for an interesting comparison with a well documented literature example. EHDP is not well absorbed and a consideration of the  $\text{pK}_a$  data ( $\text{pK}_a$ s are 1.7, 3.1, 7.5 and 11.5) (Gural *et al.*, 1985) suggest it is highly ionised at physiological pH. Michael *et al.* (1972) found absorption to be less than 10% in the rat, rabbit and monkey whilst ranging from 13.6 to 21.1% in the dog depending on age. Other investigators (Wasserman *et al.*, 1973; Gural *et al.*, 1985) have reported similar findings in animal studies with EHDP. Although Gural *et al.* (1985) found a dose dependant relationship, it appears high absorption rates at high dose levels correlated to structural alterations in the mucosal membrane. Recker and Saville (1973) using human subjects and a variety of techniques estimated absorption values ranging between 1.49% and 7.19%.



## 1.5 PRESYSTEMIC METABOLISM - THE EFFECT ON ABSORPTION

Presystemic metabolism is an aspect of gastrointestinal absorption that needs to be considered and a range of enzymes which are active in biotransformation have been described. For convenience, enzymatic metabolism of xenobiotics has been divided into two groups. Phase 1 reactions include examples such as oxidation, hydrolysis, reduction, dealkylation, and desulphuration while phase 2 reactions involve conjugation to an endogenous metabolite (eg. glycine, glucuronic acid) (Hartiala, 1973; Caldwell and Varwell Marsh, 1982).

Regarding PC, enzymatic hydrolysis to citrate is possible, *in vitro*. Accordingly, alkaline phosphatase has been used in the laboratory for analytical purposes but the assay conditions require a pH of 10.5 with a large excess of enzyme. Similarly, acid phosphatase, at pH 4.8, has been used for hydrolytic purposes in the detection of PC on TLC plates (Williams and Sallis, 1980) but again relatively large amounts of enzyme are required for phospho-hydrolysis to occur. Enzymatic metabolism, *in vivo*, has not been documented so possible *in vivo* hydrolysis within the gastro-intestinal tract would need to be explored. Clearly enzyme activity in the intestine (Renwick and George, 1989), liver (George and Shand, 1982) and lung (Routledge and George, 1982) can have a major effect on bioavailability through biotransformation of a drug species prior to entry into the systemic circulation.

To be absorbed from the intestine, a drug species needs to be resistant to chemical and enzymatic transformation in the stomach and lumen before, as indicated above, facing a complex range of biotransforming enzymes in the

intestinal epithelia. This metabolic barrier acts essentially to protect the organism against potentially toxic substances but it is sometimes a distinct barrier to convenient drug therapy. Compounds which are metabolised by intestinal tissue include pyridoxal 5'-phosphate (Middleton, 1986), chlorpromazine (Curry *et al.*, 1971), Leu-Enkephalin (Freidman and Amidon, 1991), organic nitrate esters (Posados *et al.*, 1988) and thiamin phosphates (Gastaldi *et al.*, 1988). Detailed compilations of gut metabolism are given by Routledge and Shand (1979); Caldwell and Varwell Marsh (1982).

Another aspect of drug metabolising activity is that this activity is not uniform within the intestine. This fluctuation can be local or more general, implying the existence of gradients lengthwise within the gastrointestinal tract (Renwick and George, 1989). Weiser *et al.* (1986) describe a number of epithelial enzymes which change in activity along the length of the intestine. Alkaline phosphatase for example has high duodenal activity but displays a sharply decreasing gradient towards the distal portions of the intestine. The impact of the intestinal micro flora on metabolism and absorption is also relevant (Goldman, 1982).

## 1.7 RESEARCH AIMS

The overall purpose of these studies was to investigate the effectiveness of PC when delivered by the oral route. As all studies to date have used the intraperitoneal route for testing efficacy, a comparison of the effectiveness of PC on calcification when delivered by the two administration routes was thought necessary. The need to determine the actual extent of uptake from the intestine using specialised *in vitro* and *in vivo* techniques was also envisaged.

Insights into the absorptive process thus obtained, would be expected to provide information on how to maximise the delivery of PC to the systemic circulation.

## CHAPTER 2

### INVESTIGATION OF PHOSPHOCITRATE ORAL BIOAVAILABILITY USING A CALCERGY MODEL

#### 2.1 INTRODUCTION

Previous studies on the effectiveness of PC in preventing biological calcification have utilised the intraperitoneal route of administration. The dose necessary for an effective biological response has varied depending on the site of calcification. This indicates that biological parameters may influence the biodistribution of PC, which is not unexpected given the unusual physico-chemical nature of PC. Information concerning the bioavailability of PC following oral administration is a necessary part of any pharmaceutical evaluation of this compound as absorption from the gastrointestinal tract has long been the preferred route of administration for most therapeutic agents (Ranade, 1991).

In the present chapter calcergy (Doyle *et al.*, 1979) is used as a model to compare the systemic bioavailability of PC when given orally versus an intraperitoneal injection. The method initially developed by Seyle *et al.* (1962) involves the subcutaneous injection of a dilute metal salt or oxidising agent which will induce the formation of a calcergic plaque. Seyle and Berczi (1970) list 25 compounds that can elicit this response in the rat. Accordingly a local calcergic reaction can be induced in the mature collagenous connective tissue of the dorsal fascia and several specific events associated with this process have been noted (Hirsch *et al.*, 1984). Transient hyperaemia and increased vascular permeability result within several hours. This vascular reaction has been linked

to the degranulation of mast cells which has been noted 5 min after injection (Bridges and McClure, 1972). The calcergic reaction is well established within 24 h, along with an accumulation of macrophages and multinucleated giant cells at the edges of the expanding calcific zone (Hirsch *et al.*, 1984). Ultrastructural studies (Boivin *et al.*, 1987) indicate that demineralisation begins within 15 days.

The structured nature of calcergy development indicates that it should be suitable as a model for the screening of anticalcifying agents. McClure (1979) compared the inhibitory effects of pyrophosphate and EHDP on simple calcergy and Doyle *et al.* (1979) used potassium permanganate ( $\text{KMnO}_4$ ) induced calcergy as a means of studying the effects of various drugs on hydroxyapatite crystal deposition. The latter authors used X-ray spectroscopy to demonstrate that the plaques were comprised of hydroxyapatite and Boivin *et al.* (1987) observed needle-like structures, apatite in nature, deposited between and around collagen fibrils. In the present studies  $\text{KMnO}_4$  was used to induce calcergy and reduction in calcification from treatment with PC was evaluated as a response to the presence of PC in the systemic circulation.

## **2.2 METHODS**

### **2.2.1 General reagents and equipment**

Reagents used were AR grade when possible. Buffers and solutions were made with double distilled water. Equipment used included an atomic absorption spectrophotometer (Varian techtron, model 1000) and Beckman (DU-30) spectrophotometer.

### **2.2.2 Synthesis of phosphocitrate**

PC was prepared by a combination of methods described by Tew *et al.*

(1980) and Williams and Sallis (1980). The tertiary hydroxyl group of triethyl citrate was phosphorylated using *o*-phenylene phosphochloridate, the latter reagent being synthesised by the method of Khwaja *et al.* (1970). Phosphorylation was followed by hydrogenation and base hydrolysis in the presence of  $\text{CaCl}_2$ . Final purification involved cation exchange on AG.50W-X8 followed by gradient elution with bicarbonate buffer using the anion exchanger AG.1-X8. PC which was characterised by previously published methods (Williams and Sallis, 1980; Williams and Sallis, 1981) had a molecular weight of 450.

### **2.2.3 Induction of local calcergy**

Male Hooded Wistar rats weighing 200 g were injected subcutaneously with 200  $\mu\text{l}$  of a freshly prepared  $\text{KMnO}_4$  solution (1 mg/ml). The rats were injected in two positions either side of the spine in the interscapular region. The calcific plaques were allowed to develop for a period of 10 days after which the rats were killed by anaesthetic overdose. The plaques were excised for later analysis. In all respects the animals remained healthy following the treatment and attained the same body weight at the end of the 10 day period.

### **2.2.4 Analysis of calcific plaques**

#### **2.2.4.1 Treatment of plaques**

Immediately after excision the plaques were blotted dry on tissue paper, plunged into liquid nitrogen and then immediately weighed. Storage was at  $-20^\circ\text{C}$ . Plaques were ground into a powder using an aluminium mortar and pestle which was kept cold in a bath of liquid nitrogen. Aliquots of powder (15-25 mg) were quickly weighed and placed in glass vials. A solubilisation mixture comprising of 70% perchloric acid (200  $\mu\text{l}$ ) and 30% hydrogen peroxide (400

$\mu$ l) was added before fitting the vials with acid resistant teflon caps and digestion at 80°C for 2 h. After cooling, the contents of each flask was made up to 5 ml with water.

#### **2.2.4.2 Estimation of calcium**

The calcium content of plaques was determined using atomic absorption spectrophotometry. For measurement, aliquots of plaque digests (0.5 ml) were diluted to 10 ml with a lanthanum chloride solution (50,000 ppm  $\text{La}^{3+}$ ). Standards were in the range 0-2.5  $\mu\text{mol Ca}^{2+}$  / ml).

#### **2.2.4.3 Estimation of phosphate**

The phosphate content of plaques was determined using the method of Fiske and SubbaRow (1925). Any interference with the assay due to the presence of the tissue oxidising mixture was prevented by the addition of sodium sulfite (100  $\mu$ l, 33%  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ ). Standards in the range 0-1.0  $\mu\text{mol PO}_4$  were used.

#### **2.2.4.4 Histological evaluation of plaques - von Kossa stain**

Plaques were fixed in 10% phosphate buffered formol-saline at pH 7.4 for 24 h. The tissue was then dehydrated using ascending grades of ethyl alcohol (50-100%) and cleared in xylene before being infiltrated and embedded with paraffin wax. Sections (5  $\mu\text{m}$ ) were cut using a microtome and applied to slides which were deparaffinised in xylene. The slides were treated with 5% silver nitrate and exposed to a bright light (60 W) for 60 min. After rinsing in water, immersion in 3% sodium thiosulphate and again rinsing in water, the slides were counterstained with van Geison stain. Finally sections were dehydrated, cleared and mounted.

### 2.2.5 Administration of PC

Trials involving inhibition of calcification by various routes of administration were made. Compounds were administered by gavage (oral), intraperitoneal or subcutaneous injection. Rats were divided into groups (6-8 rats/ group) and administered the test substance twice daily (9.00 am and 5.00 pm). Rats designated as a control group received an equivalent volume of isotonic saline alone and it has been demonstrated previously that citrate and phosphate do not have an effect on *in vivo* calcification when compared to PC (Ward *et al.*, 1986). On day one of each trial, rats were dosed with PC 1 h before administration of  $\text{KMnO}_4$ .

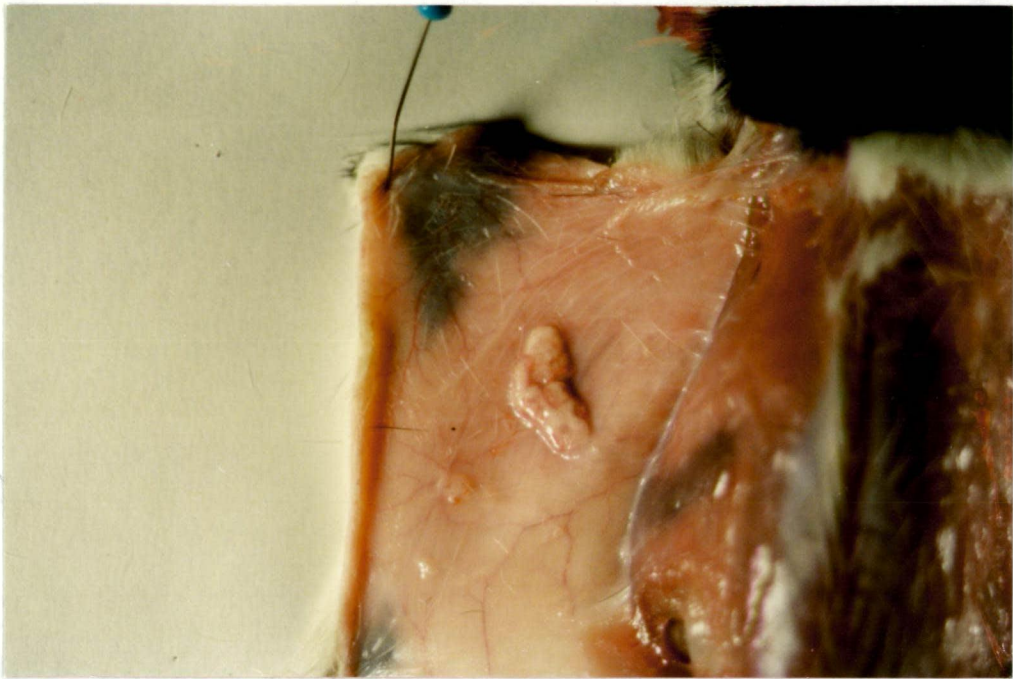
### 2.2.6 Evaluation of data

Plaque differences were examined in several ways. In each experimental group, plaques were weighed and analysed for calcium and phosphorus. Data was compared using the single tailed student t-test. Calcium/phosphorus ratios were compared for some experimental groups.

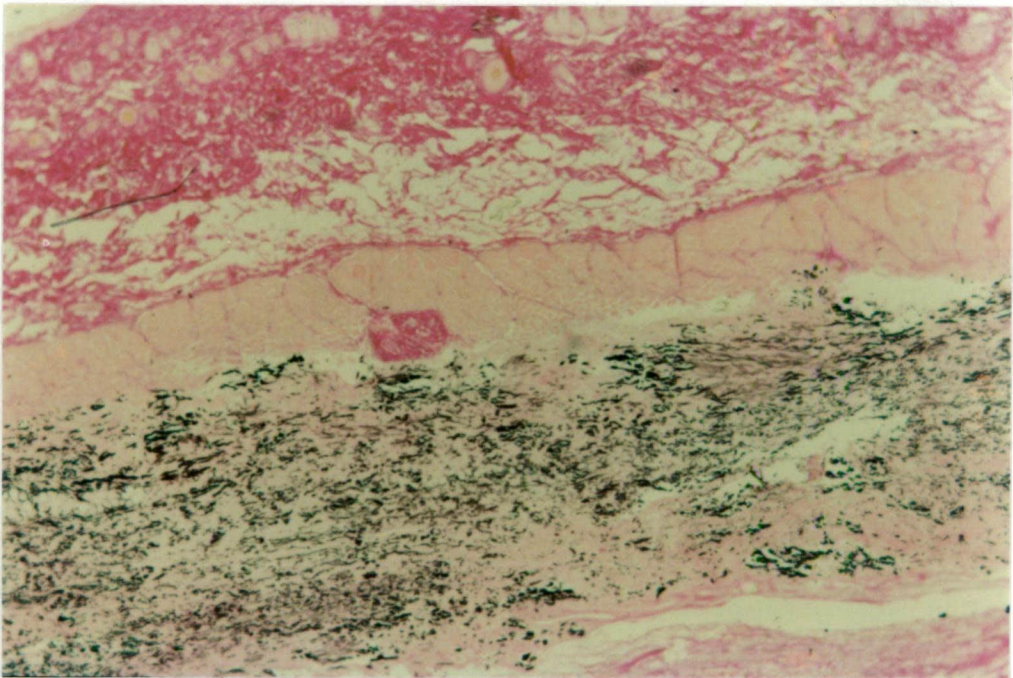
## 2.3 RESULTS

A subcutaneous injection of  $\text{KMnO}_4$  induced the formation of a calcergic plaque (Figure 2.1). The plaques were demonstrated to contain calcium and phosphate by analysis of these parameters and by staining using the von Kossa method (Figure 2.2). Typically the plaques were round to oval in shape and of a milky white appearance. During early studies, plaque dimensions were measured. The average dimensions for plaques examined for this parameter were 16 mm by 10 mm ( $n = 16$ ). Plaque thickness ranged between 1-3 mm.





**Figure 2.1** Photograph of a plaque



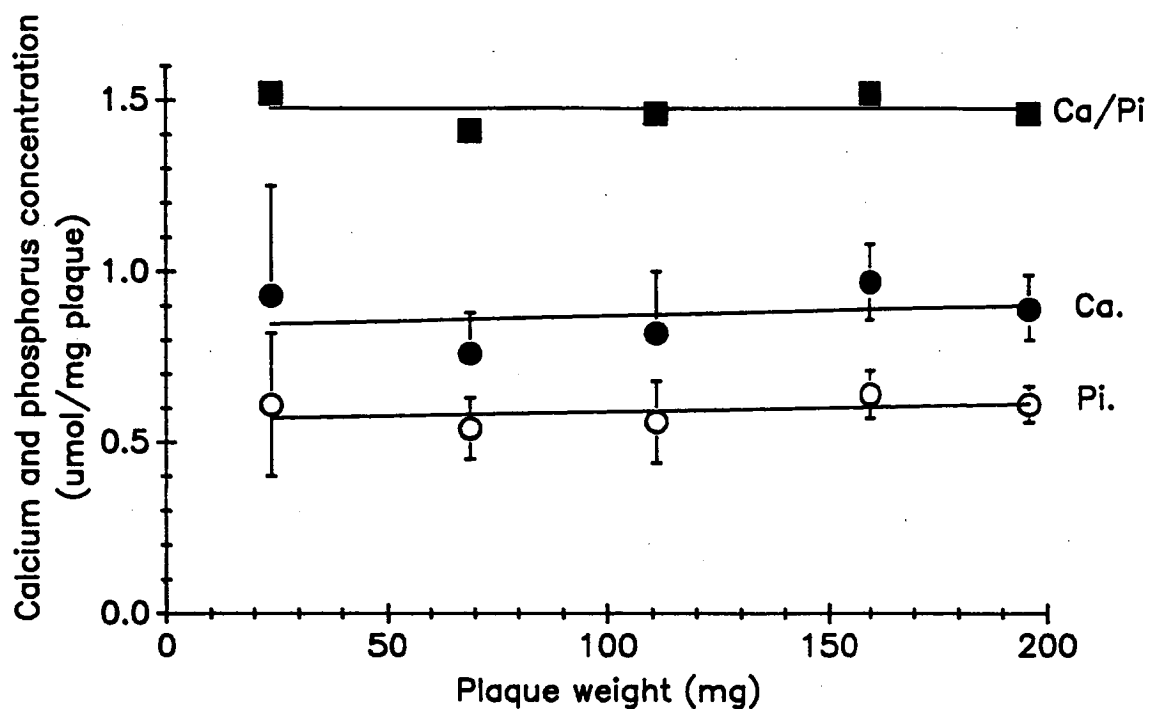
**Figure 2.2** Photograph showing a transverse section of plaque with skin attached and stained using the von Kossa technique.

Differences in plaque formation as a result of inhibitor administration have been expressed as changes in mean total weight. Analyses of plaque calcium and phosphorus were also performed. Changes in the total calcium and phosphorus content of plaques was found to mirror the changes in plaque weight. The concentration of calcium and phosphorus (per mg plaque) was calculated and found to be similar in all plaques regardless of plaque weight (Figure 2.3). Calcium/phosphorus ratios were consistent with the precipitated calcium salt being hydroxyapatite.

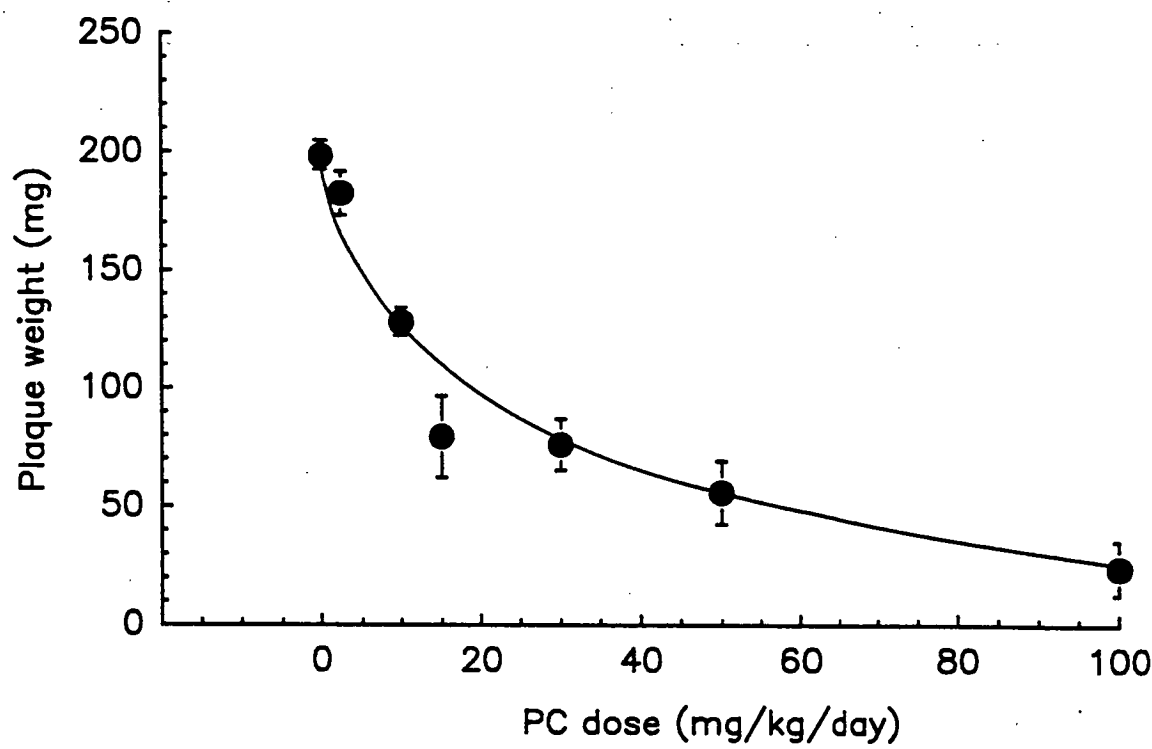
The response of plaque weight to the intraperitoneal injection of PC was determined at six dose levels. Plotting mean response versus dose of PC yielded a curve (Figure 2.4). When rats were untreated, the mean value for plaque weight was 198 mg. Compared to the control group all doses except the lowest were highly significant ( $p < 0.0005$ ). The lowest dose (2.5 mg/kg/day) was marginally outside the significance level of  $0.025 < p < 0.05$ . At 10 mg/kg/day, PC reduced plaque weight by 35%, increasing to 72% at 50 mg/kg/day and 88% at 100 mg/kg/day.

Figure 2.5 shows the effect of intraperitoneal PC on the total calcium and phosphorus content of plaques at three doses. When PC was given at 50 mg/kg/day the total calcium and phosphorus content of plaques was reduced by 75% ( $p < 0.0005$ ) and 76% ( $p < 0.0005$ ) respectively. The reduction in plaque calcium and phosphorus increased to 90% and 91% respectively at the highest dose of 100 mg/kg/day. The lowest dose (2.5 mg/kg/day) did not produce statistically significant changes in plaque calcium and phosphorus ( $0.3 < p < 0.4$ ).

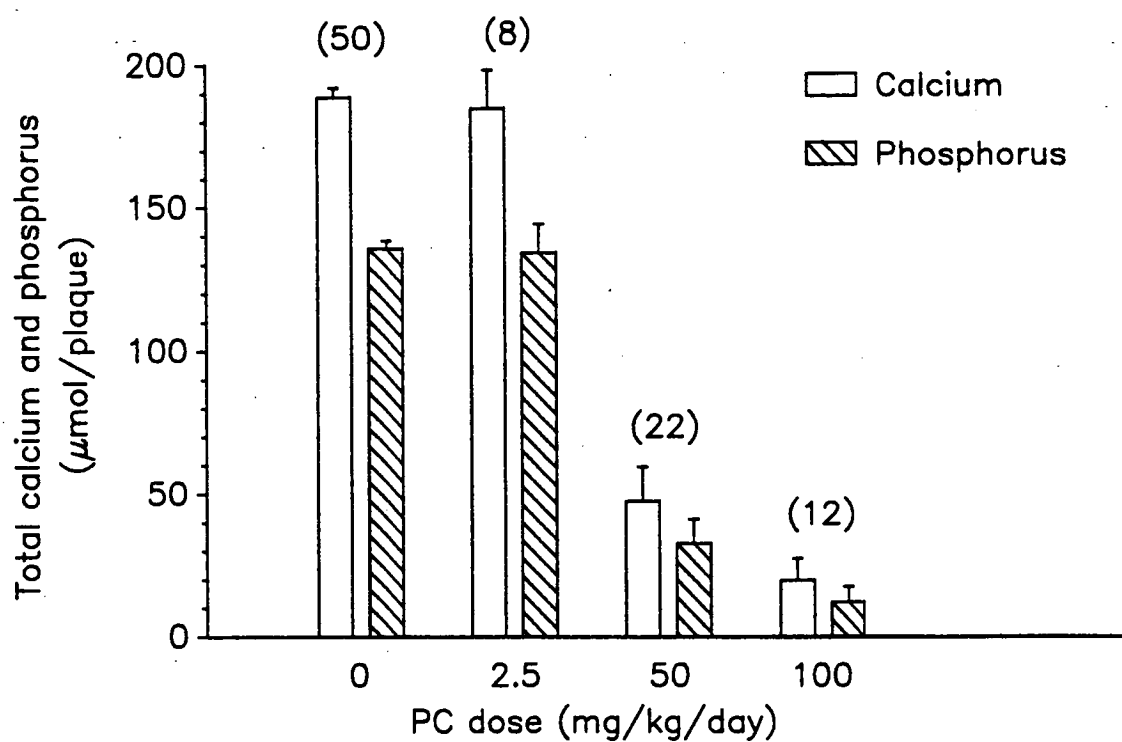
The response of plaque weight to oral dosed PC was measured when PC was administered at 100 mg/kg/day and 450 mg/kg/day and is presented in



**Figure 2.3** The concentration of plaque calcium and phosphorus and calcium/phosphorus ratio in plaques as a function of plaque weight. As indicated in section 2.2.3 body weight was the same for all animals. Data represents mean  $\pm$  1 S.D. (n=5-14).



**Figure 2.4** A dose response curve, plotted by linear regression ( $r=0.97$ ), showing the effect of intraperitoneal administered PC on plaque weight. Control rats received an equivalent volume of 0.9% saline by intraperitoneal injection. Data represents mean  $\pm$  SEM. N values refer to the number of plaques and are shown in brackets for each dose; 0 (50), 2.5 (22), 10 (14), 15 (18), 30 (16), 50 (22), 100 (12).



**Figure 2.5** The effect of PC administered by intraperitoneal injection on the total calcium and phosphorus content of plaques. Data represents mean  $\pm$  SEM. N-values are in parentheses.

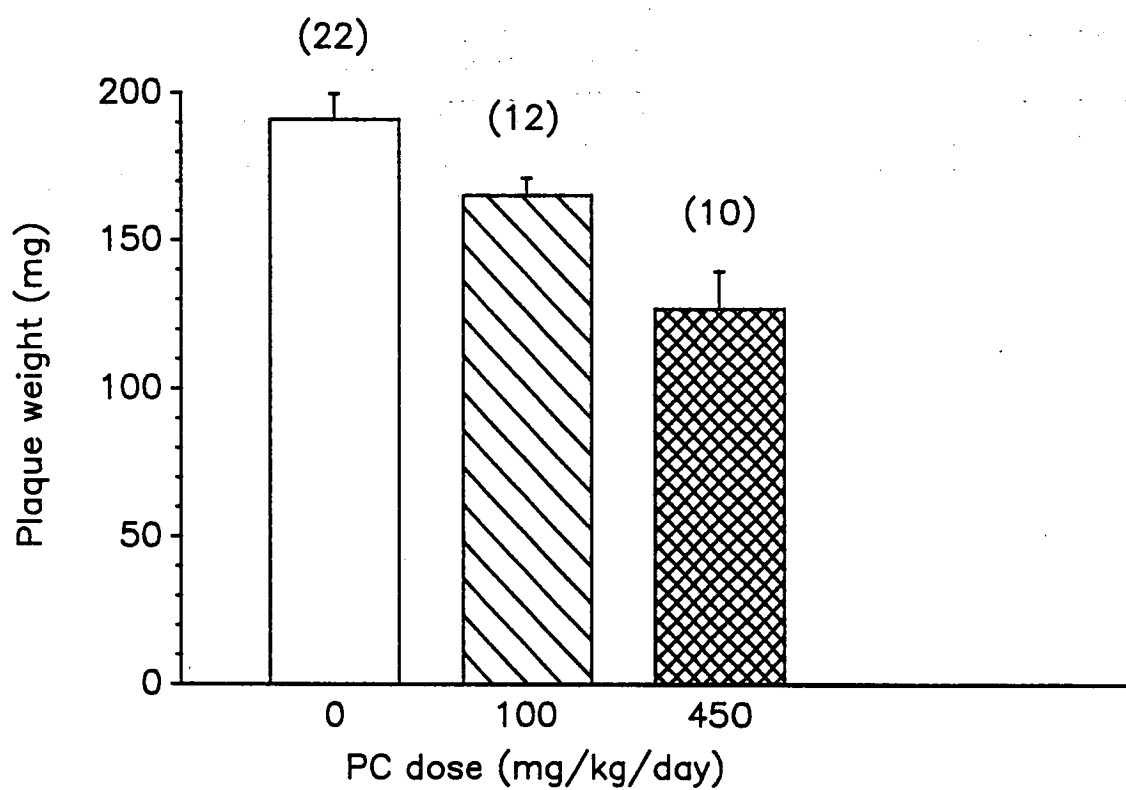
Figure 2.6. The data for reductions in total plaque calcium and phosphorus levels at the two doses is presented in Figure 2.7. A statistically significant reduction in plaque weight of 13% ( $0.01 < p < 0.025$ ) was obtained when PC was administered orally at 100 mg/kg/day. Reductions in plaque calcium of 10% ( $0.10 < p < 0.15$ ), and plaque phosphate of 6% ( $0.20 < p < 0.25$ ) were not significant. The measured plaque parameters were reduced by 34% ( $p < 0.0005$ ), 30% ( $0.0005 < p < 0.005$ ) and 24% ( $0.005 < p < 0.01$ ) respectively at the 450 mg/kg/day dose level.

The equivalent intraperitoneal dose that corresponded to a 34% reduction in plaque weight, as observed for the oral 450 mg/kg/day dose, was approximately 10 mg/kg/day. This represented a difference of 45 times when the two routes of administration were compared.

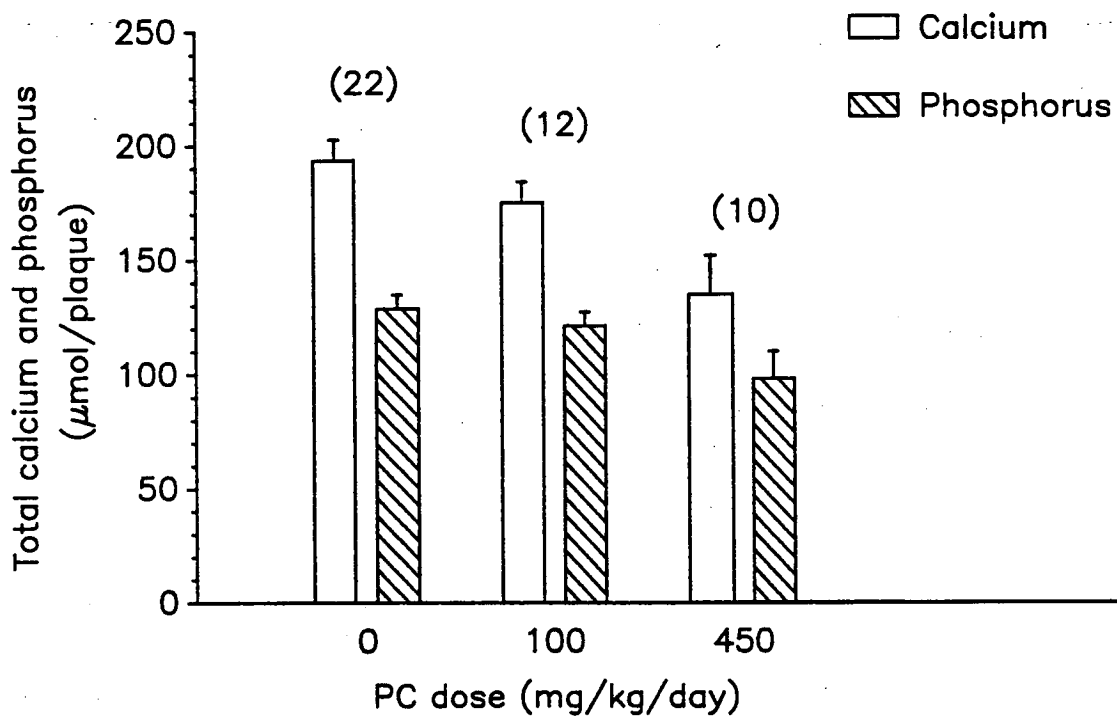
Administration of PC subcutaneously at the site of  $\text{KMnO}_4$  injection resulted in complete prevention of plaque formation. However the development of haematomas at the injection site was apparent following daily subcutaneous administration of PC.

## 2.4 DISCUSSION

The presence of hydroxyapatite in plaques formed after administration of dilute metal salts has been previously established (Doyle *et al.*, 1979; Boivin *et al.*, 1987). The present data confirms the validity of this model as analysis for calcium and phosphate in plaques indicated the presence of these elements in a ratio typical of hydroxyapatite. Trials with PC resulted in significant reductions in plaque weight following intraperitoneal administration but the response to oral delivery of PC indicated reduced bioavailability. In fact orally, at least



**Figure 2.6** The effect of orally delivered PC on plaque weight. All rats were dosed by gavage and control rats received an equivalent volume of 0.9% saline. Data represents mean  $\pm$  SEM. N-values are in parentheses.



**Figure 2.7** The change in total calcium and phosphorus content of plaques following oral administration of PC. Data represents mean  $\pm$  SEM. N-values are in parentheses.



45 times the dose of PC had to be administered to reach an equivalent potency compared to an intraperitoneal dose. This highlights a problem in the passage of PC from the intestinal lumen to the systemic circulation.

Previous studies, using a range of models, have demonstrated the overall effectiveness of PC in preventing unwanted calcification following intraperitoneal administration. Treatment with intraperitoneally injected PC in an infection stone forming model reduced stone weight by 73% (Sallis *et al.*, 1988). This compares with the 72% reduction in calcergic plaque weight obtained with an equivalent intraperitoneal dose of PC. Considerably lower doses (2.25 mg/kg/day) were needed to prevent dystrophic calcification of aortic segments transplanted to the peritoneal wall of rats (Shankar *et al.*, 1984b). This data supports the indications obtained here that the effectiveness of PC can be influenced by the route of administration.

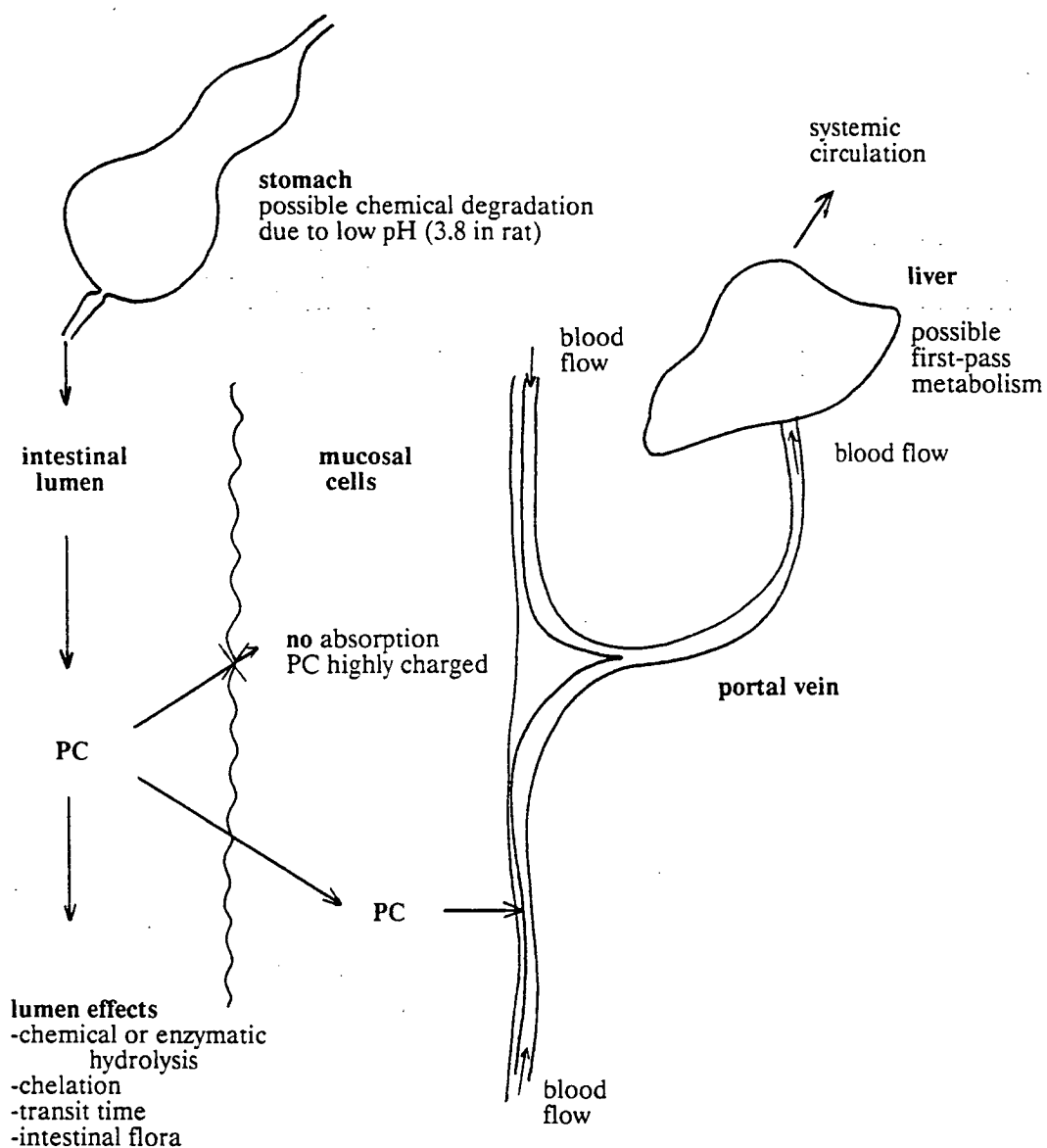
The distribution of PC into body compartments following non-targeted delivery probably means that only small amounts of PC actually reach the calcification site. This idea would seem to be verified as subcutaneous administration of PC (50 mg/kg/day) near the site of plaque formation completely inhibited the calcium salt deposition. Further studies investigating the dose response to subcutaneously administered PC might be useful in determining the real ability of this compound to prevent calcergy. This was not appropriate in this study as the model was primarily used to examine changes in bioavailability following oral administration.

In these trials PC was delivered by gavage, leaving several factors as possible reasons for the reduced systemic effect of PC following oral delivery.

Figure 2.8 illustrates some potential limiting factors that might prevent the absorption of PC into the bloodstream. Chemical and enzymatic hydrolysis in the stomach and/or intestinal lumen might reduce the availability of PC at the mucosal surface. The highly charged nature of the PC molecule might prevent passage across the mucosal barrier. In relation to this, it is known that high charge can effect the membrane permeability of a compound and hence limit transport (Schanker, 1971). Other factors which can influence absorption (Renwick, 1982) include chelation to other compounds, intestinal mobility, gut transit time and exposure to intestinal microflora.

First-pass metabolism in intestine and liver would reduce the amount of PC available within the systemic circulation. Enzymes which could theoretically hydrolyse PC are the alkaline (Fernley, 1971) and acid (Hollander, 1971) phosphatases. These enzymes have a ubiquitous tissue distribution and catalyse the hydrolysis of a wide variety of phosphate esters. Alkaline phosphatase is a membrane bound enzyme with high activity in gut tissue. However, although degradation of the PC molecule by phosphoryl esterase action has been considered by some authors (Reddi *et al.*, 1980; Williams and Sallis, 1981) no definitive evidence has arisen from these investigations to support this notion. Interestingly, Shankar *et al.* (1984a) found that PC effectively inhibits calcium uptake by matrix vesicles, despite the presence of high alkaline phosphatase activity. Additionally, the active site in alkaline phosphatase which contains  $\text{Zn}^{2+}$  is sensitive to the charged nature of potential substrates (Fernley, 1971). Altogether these factors make it unlikely that this enzyme will have a marked effect on PC *in vivo*.

Despite speculation concerning uptake and metabolism, PC has been demonstrated to be partially effective in preventing calcification when delivered



**Figure 2.8** A simple schematic representation of possible barriers to PC absorption from the gastro-intestinal tract.

orally. The limited systemic effect observed then indicates the need for more extensive investigations of the absorption process at the gut site. With more specific information, insights might be gained leading to appropriate formulations for improving the oral delivery of PC.

## CHAPTER 3

### STUDIES ON PC ABSORPTION FROM THE GUT USING IN VITRO EVERTED SACS AND IN SITU INTESTINAL LOOPS

#### 3.1 INTRODUCTION

As indicated in chapter 2 the anticalcifying activity of PC and hence its bioavailability appeared reduced following oral administration as compared to the intraperitoneal route. Whilst this information was intrinsically valuable, the need for data concerning the intestinal transport of PC was apparent. To help evaluate the likelihood of being able to successfully administer PC orally, experiments were conceived which might help gain an understanding of the absorption process concerning PC.

Numerous models for studying gastrointestinal absorption have been reported in the literature (Houston and Wood, 1980; Csaky, 1984; Ings, 1984). In the early stages of investigating intestinal drug absorption the two most common strategies for experimentation are (1) *in vitro* methodology which involves the use of isolated gastrointestinal tissue and (2) *in situ* techniques which involve experiments on anaesthetised animals with an intact vascular network.

According to Barr and Riegelman (1970) *in vitro* techniques can provide an indication of relative permeability and also information on the capacity of mucosal enzymes to metabolise drugs. A wide range of *in vitro* techniques exist and include everted sacs (Wilson and Wiseman, 1954), everted intestinal ring

slices (Stewart *et al.*, 1986), isolated brush border membrane vesicles (Kessler *et al.*, 1978), and isolated enterocytes (Kimmich, 1970; Pinkus, 1981).

In order to gain insights into appropriate methodology for studying PC absorption, a study of the literature concerning citrate absorption was considered useful as although PC is structurally a unique compound, it nevertheless does have some structural homologies with citric acid. From early studies (Martensson, 1940) and more recently those of Behnke *et al.* (1966) it is clear that citrate can be recovered in the blood after perfusing citric acid in the intestine. Browne *et al.* (1977) reported using the everted intestinal sac model to study the gastrointestinal absorption of citric acid. Houston and Wood (1980) in a review on gastrointestinal absorption of drugs suggested that the everted sac model is particularly suitable for exploratory and screening work. The technique, with the creation of an artificial serosal chamber and exposure of the mucosal cells to a relatively large volume of oxygenated and mixed nutrient medium, allows permeability and any potential first pass effects to be investigated.

In respect to *in situ* techniques, two methods, termed closed loops and perfused loops, are commonly used. Levine and Pelikan (1961) have described a relatively simple version of the closed loop which involves the surgical creation of a drug filled closed intestinal loop while the animal is lightly anaesthetised. The animal is then allowed to recover from the anaesthetic and the loop contents are measured at a designated time afterwards. Perfused loops involve the simulated passage of drug through the segment of gut being studied allowing samples to be obtained during the time course of the experiment.

Whilst elaborate *in situ* methodologies do exist (Hayton and Levy, 1972), Houston and Wood (1980) have described the closed loop method detailed by Levine and Pelikan as the most physiological of the *in situ* models. The procedure allows the animals to be ambulatory within 5 min of completing the cannulation procedure, hence minimising any effects of anaesthesia on the absorption process. The purpose of the studies reported in this chapter were to measure the *in vivo* uptake of PC from the lumen along with the *in vitro* passage of PC across the gut and any potential metabolism.

## **3.2 METHODS**

### **3.2.1 General reagents and equipment**

Enzymes were obtained from Boehringer Mannheim (Australia). Equipment used included a Metabolyte shaking water bath (New Brunswick Scientific Co.) and a Ratio Fluorimeter (Beckman).

### **3.2.2 Analysis of PC and citrate**

Analysis of PC was according to the method described by Williams and Sallis (1980) using a coupled enzyme assay. Following preincubation of PC with alkaline phosphatase, malate dehydrogenase, NADH and citrate lyase were added and the conversion of NADH to  $\text{NAD}^+$  was followed fluorometrically. The reaction sequence for this assay is PC→citrate→oxaloacetate→malate.

### **3.2.3 Everted sac preparation**

The method was similar to that described by Browne *et al.* (1978) based on an original description by Wilson and Wiseman (1954). Male Hooded Wistar rats weighing 200 g were killed by cervical dislocation. The small intestine was exposed by a midline incision and a 10 cm section beginning approximately 5 cm

posterior to the pyloric sphincter was surgically isolated and dissected away from the mesentery.

The segment was washed free of particulate matter with isotonic saline at room temperature and then everted on a glass rod (O.D. 5.0 mm). The everted sac was closed at one end with a silk ligature, then filled with 1.0 ml of Krebs-Henseleit buffer containing 5 mM glucose (KHG; comprising 0.154 M NaCl (100 pts), 0.154 M KCl (4pts), 0.11 M  $\text{CaCl}_2$  (3pts), 0.154 M  $\text{KH}_2\text{PO}_4$  (1pt), 0.154 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (1pt), 1.3%  $\text{NaHCO}_3$  (21 pts), 0.130 mM glucose (5pts); Dawson, 1986). Once filled the segment was closed at the other end with a silk ligature.

The sacs were incubated in 25 ml KHG in 50 ml Erlenmeyer flasks. Incubations were performed at 37°C in a shaking water bath (80 oscillations / minute) under a gas phase of 5%  $\text{CO}_2$ / 95%  $\text{O}_2$ . The buffer was prepared fresh each day from stock solutions, maintained at 37°C and equilibrated with the same gas phase prior to being used for incubations.

The fluid contained within the artificially created sac, and in contact with the serosal surface of the gut segment, was called serosal fluid and the fluid bathing the intestinal mucosa was termed the mucosal fluid. Test substances (PC and citrate) were added to the mucosal fluid in known concentrations according to the design of the experiment. Serosal fluid volume changes were determined by weight.

#### **3.2.4      In situ loop preparation**

Male Hooded Wistar rats weighing 200 g were lightly anaesthetised with ether and then maintained under halothane anaesthesia (fluothane, ICI



Australia) using a Boyles Apparatus (Fluotec MK. 2, Cyprane Ltd. UK.). The anaesthetic conditions were controlled at 1.5% halothane and 3000cc/min oxygen. The abdominal cavity was opened with a 1.5 cm midline incision. The appropriate region of intestine was externalised and a 10 cm section, starting approximately 5 cm posterior to the pyloric sphincter, was identified.

A small incision was made 0.3 cm beyond each end of the 10 cm sections. A glass cannula was inserted into the distal end and held in place with a silk ligature. The segment was then rinsed free of its contents with isotonic saline at 37°C. Air was gently forced from the loop before ligation of the proximal end. PC (22.5  $\mu$ mol) was introduced (via the cannula) as a solution in isotonic saline. The distal end was then carefully ligated as the cannula was withdrawn. The intestines which had been kept warm with a saline moistened pad (37°C) during surgery were returned to the abdominal cavity which was then sutured close before the rat was allowed to recover from the anaesthetic. The rats were aided in the maintenance of body temperature post-operatively through the use of a heat lamp.

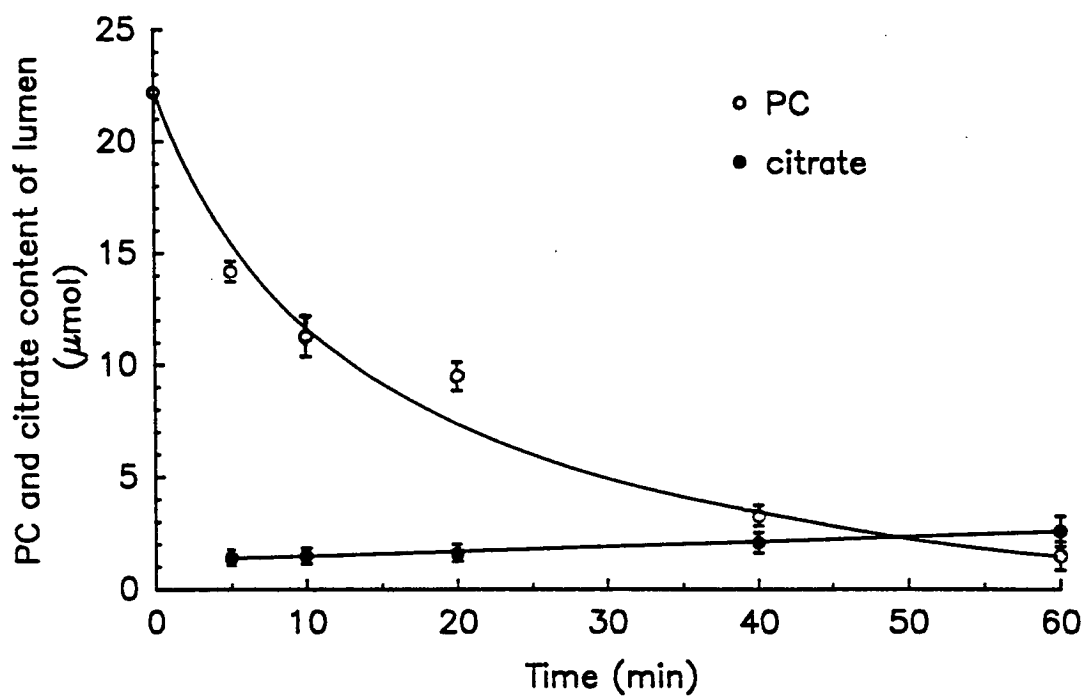
At timed intervals following the introduction of PC the rats were killed by cervical dislocation, the abdominal cavity opened and the segment quickly removed. The contents of the closed loops were retained and the loop twice washed with ice-cold distilled water. The washings were pooled with the original contents and the combined total volumes determined by weight. Solutions for assay were either stored frozen or maintained at 4°C prior to an immediate assay. PC and citrate contents were determined using the coupled enzyme assay (3.2.2).

### 3.3 RESULTS

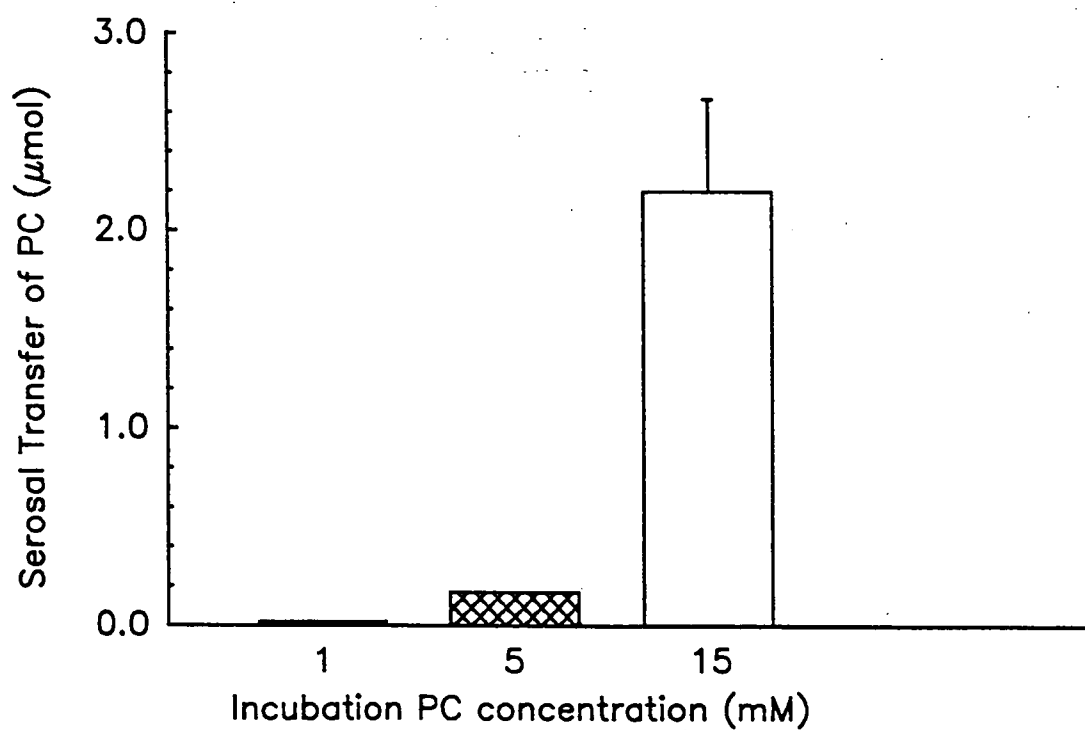
Disappearance of PC from the intestinal lumen using an *in situ* technique was observed (Figure 3.1). The rate of disappearance of PC was initially rapid but slowed over the 60 min time course of the experiment. Examination of the data revealed that this was not a first order process. Additionally a steady but small linear increase in lumen citrate was noted. During the initial 10 min the lumen PC content was reduced from 22.2  $\mu\text{mol}$  (22.2mM) to 11.3  $\mu\text{mol}$ . This represents disappearance at the rate of 10.9  $\mu\text{mol}/10\text{ cm}/10\text{ min}$ . At 60 min, lumen PC was reduced to 1.5  $\mu\text{mol}$  which represents an overall rate of disappearance of 2.0  $\mu\text{mol}/10\text{ cm}/10\text{ min}$  during the final 50 min. Lumen citrate was found to increase from 1.4  $\mu\text{mol}$  at 5 min to 2.6  $\mu\text{mol}$  at 60 min.

Figure 3.2 shows the serosal transfer of PC after incubation of everted sacs with different concentrations of PC. In the presence of 1 mM PC, no PC was detected in the serosal fluid. Incubation with 5 mM PC resulted in transfer of 0.17  $\mu\text{mol}/10\text{ cm sac}/\text{h}$ . Transfer increased to 2.20  $\mu\text{mol}/10\text{cm sac}/\text{h}$  when incubations were in the presence of 15 mM PC. Additional to the serosal transfer of PC, it was noted that citrate transfer also occurred (data not shown) and it was apparent that the ratio of PC to citrate transferred increased as the initial incubation concentration of PC in the mucosal fluid increased.

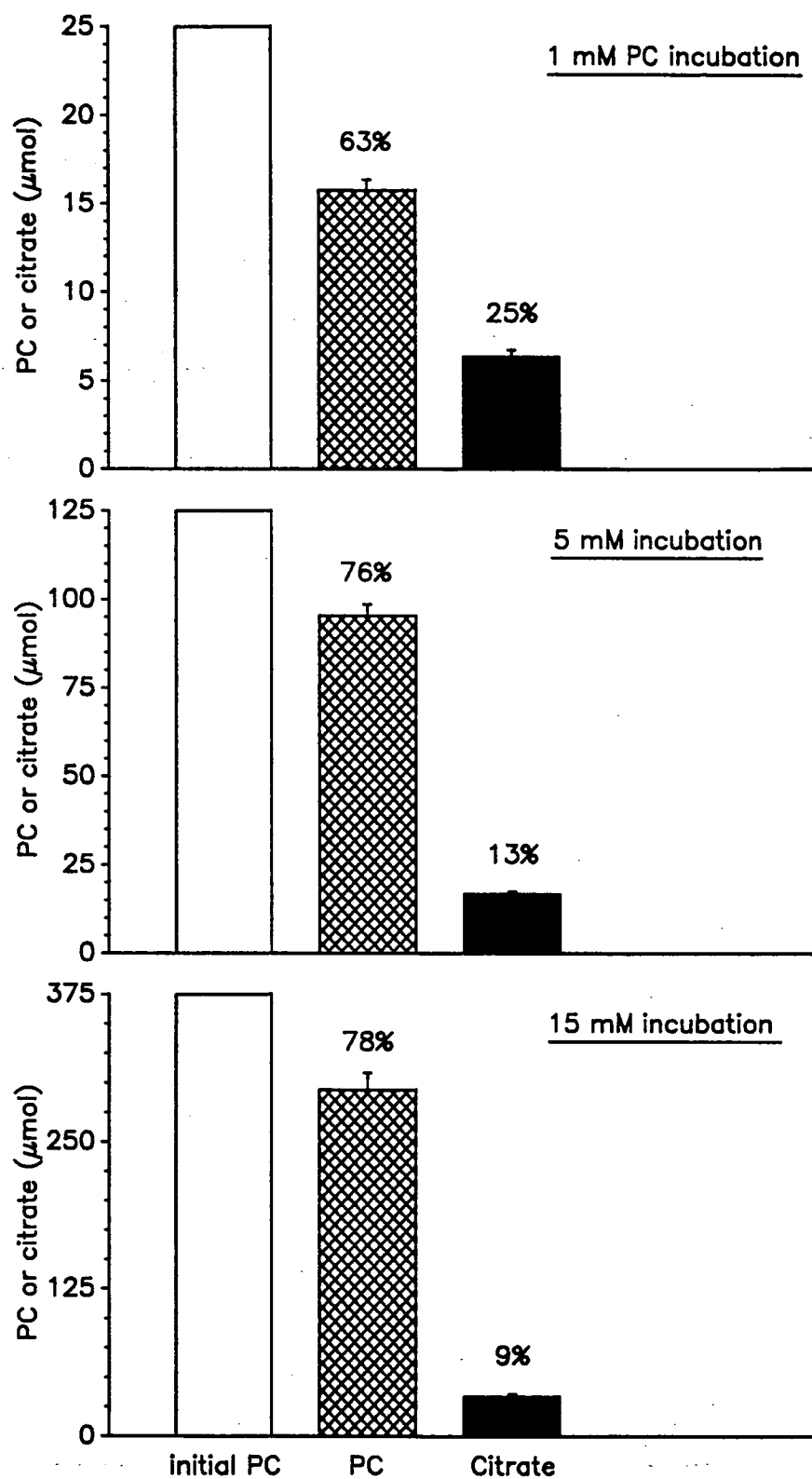
Another interesting aspect noted from the everted sac experiments was obtained from examination of the data presented in Figure 3.3 which shows the content of the mucosal fluid after 1 h incubations. At the lowest initial concentration of PC (1 mM) 63% of PC remained after 1 h. On a molar basis 25 % (6.4  $\mu\text{mol}$ ) of the initial PC present in the mucosal fluid was found as citrate



**Figure 3.1** Changes in PC and citrate content of isolated loops during an *in situ* perfusion. Data represents mean  $\pm$  SEM. (n=3)



**Figure 3.2** Appearance of PC in the serosal fluid of everted sacs. Incubations were in KHG buffer for a period of 1 h and the data is the total amount of PC transferred during that period. Data represents mean  $\pm$  SEM. (n=6)



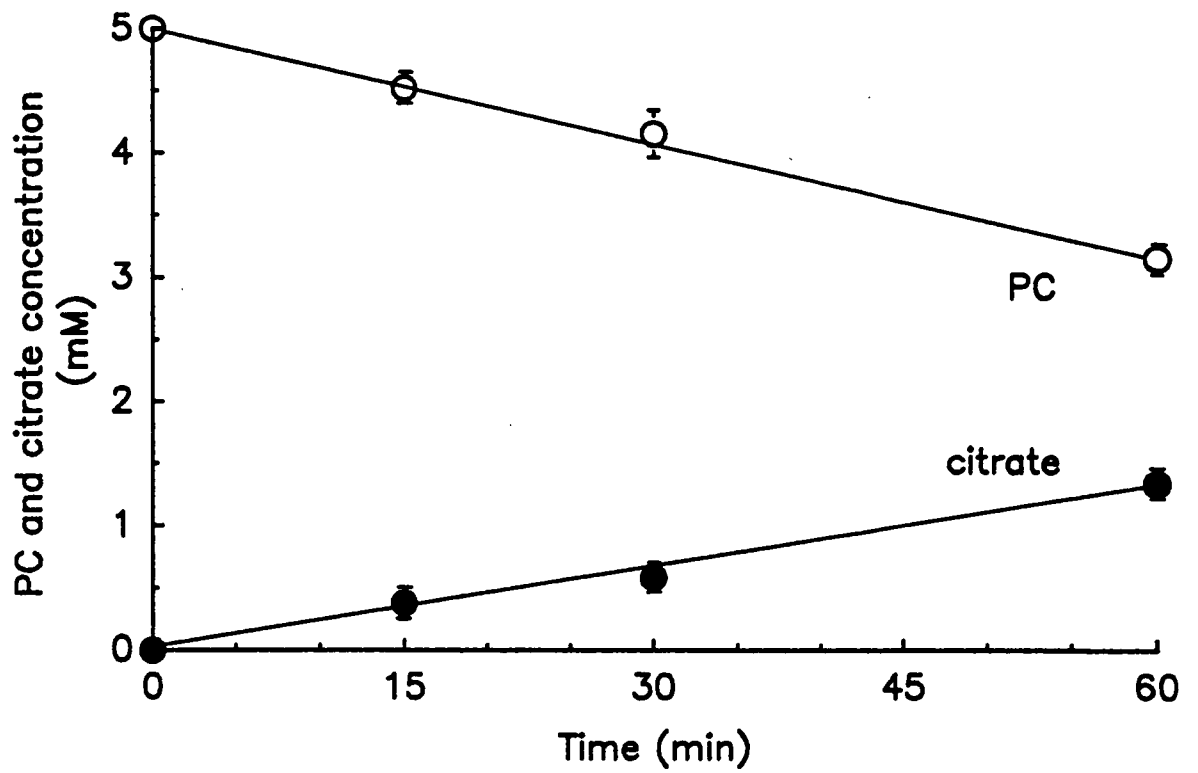
**Figure 3.3** The PC and citrate content of mucosal fluid in everted sacs following incubations. The values expressed as a percentage are on a molar basis and refer to the amount of PC and citrate remaining after 1 h compared to the PC present at time zero. Data represents mean  $\pm$  SEM. (n=6)

after the 1 h incubation and data from control experiments where sacs were incubated in buffer alone indicated that no citrate or PC was found in the mucosal fluid after 1 h. Increasing the concentration of PC in the incubation fluid resulted in increased proportions (76% for 5 mM and 78% for 15 mM) of PC remaining after 1 h.

The proportion of citrate present in the mucosal fluid after incubations, decreased as the concentration of PC in the initial incubation buffer increased. Incubation with 5mM PC resulted in 13% (16.7  $\mu$ mol) of the initial PC being found as citrate after 1 h. Incubation with 15 mM PC showed 9% (33.9  $\mu$ mol) citrate after 1 h. This corresponded to 256 nmol citrate/  $\mu$ mol PC/ h appearing during the 1 mM PC incubation and 134 and 90 nmol citrate /  $\mu$ mol PC / h respectively for the 5 and 15 mM PC incubations. Additionally the disappearance of mucosal PC and appearance of mucosal citrate was examined with respect to time using 5 mM PC incubations. The results (Figure 3.4) showed that the concentration of PC decreased in a linear fashion whilst at the same time the citrate concentration increased linearly.

### 3.4 DISCUSSION

Data which emerged from the *in situ* investigations indicated that PC was taken up from the rat intestine. The disappearance was initially rapid but slowed as the amount of PC within the loop decreased. This could have been a function of the amount of PC available at the gut surface for transfer. Limited amounts of citrate also accumulated in loops thus indicating that some metabolism of PC to citrate probably occurred. However the extent of intraluminal metabolism of PC could not be determined as it was not feasible to obtain an accurate estimation of citrate uptake using this technique.



**Figure 3.4** Changes in the concentration of PC and citrate in the mucosal fluid of everted sacs during incubations. The initial incubation medium contained 5 mM PC. Data represents mean  $\pm$  SEM. (n=3)

Studies with the everted sac model confirmed PC transfer across the mucosal barrier. However as opposed to the minimal amounts of citrate observed intralumenally with the *in situ* technique, large amounts of citrate readily accumulated in the mucosal fluid during everted sac incubations. This accumulation appeared to be rate limiting as the amount of citrate as a proportion of the initial amount of PC present decreased with increasing incubation concentrations of PC. One possible explanation for this decrease is that enzyme sites available for the hydrolysis of PC were partially saturated.

Compared to the amount of PC that disappeared from the *in situ* lumen preparation, only minimal amounts of PC were found to appear in the serosal fluid of everted sacs. The viability of the everted sac preparations was confirmed by comparing data for citrate transport to that previously obtained by Browne *et al.* (1977). Incubations with 1 mM citrate showed a comparable rate of serosal citrate accumulation to that obtained by the earlier study.

The apparent differences between the two methods may be explained by consideration of the nature of the two techniques. Physiologically, the absorption process is quite complex and ultimately depends on an intact circulation. The everted sac method is an *in vitro* technique and hence lacks the microcirculation which would normally remove absorbed substances. Compounds must pass through the epithelial cell layer, the submucosa, external muscle layers and serosal tissue of the gut wall before entering the artificially created serosal chamber. This may also explain why only limited transfer to serosal fluid was observed using the everted sac model. McElnay *et al.* (1986) studied five drug absorption models including everted sacs and found that this model demonstrated comparatively small absorption of test compounds.



Concerning metabolism of PC to citrate, both techniques used here provide some evidence of potential first-pass effects. The accumulation of citrate in the lumen of *in situ* loops though minimal was nevertheless suggestive of intraluminal metabolism. However it is possible that citrate reenters the lumen following absorption and metabolism of PC intracellularly. Regarding the everted sacs, some authors (Gibaldi and Grundhofer, 1972; Levine *et al.*, 1970) have claimed that everted sac preparations can lose structural integrity during the timecourse of an experiment. This loss of integrity, which is inherent in the method, may result in the release of hydrolytic enzymes which would not otherwise have been able to effect PC.

Summarising, both techniques indicated that PC was removed from the gut lumen. Further to this PC was found in the serosal fluid of everted sacs thus demonstrating actual transfer. Also an examination of the everted sac data showed that a steady accumulation of citrate occurred and thus revealed the possibility of a first pass effect. To further understand the absorption process, other more detailed investigations need to be instigated. Certainly, the appearance of PC in the circulation following uptake from the lumen would be indicative of some transport occurring and is therefore an important aspect. Hepatic-portal sampling during an intestinal perfusion may help provide additional data for understanding the problems associated with PC uptake.

## CHAPTER 4

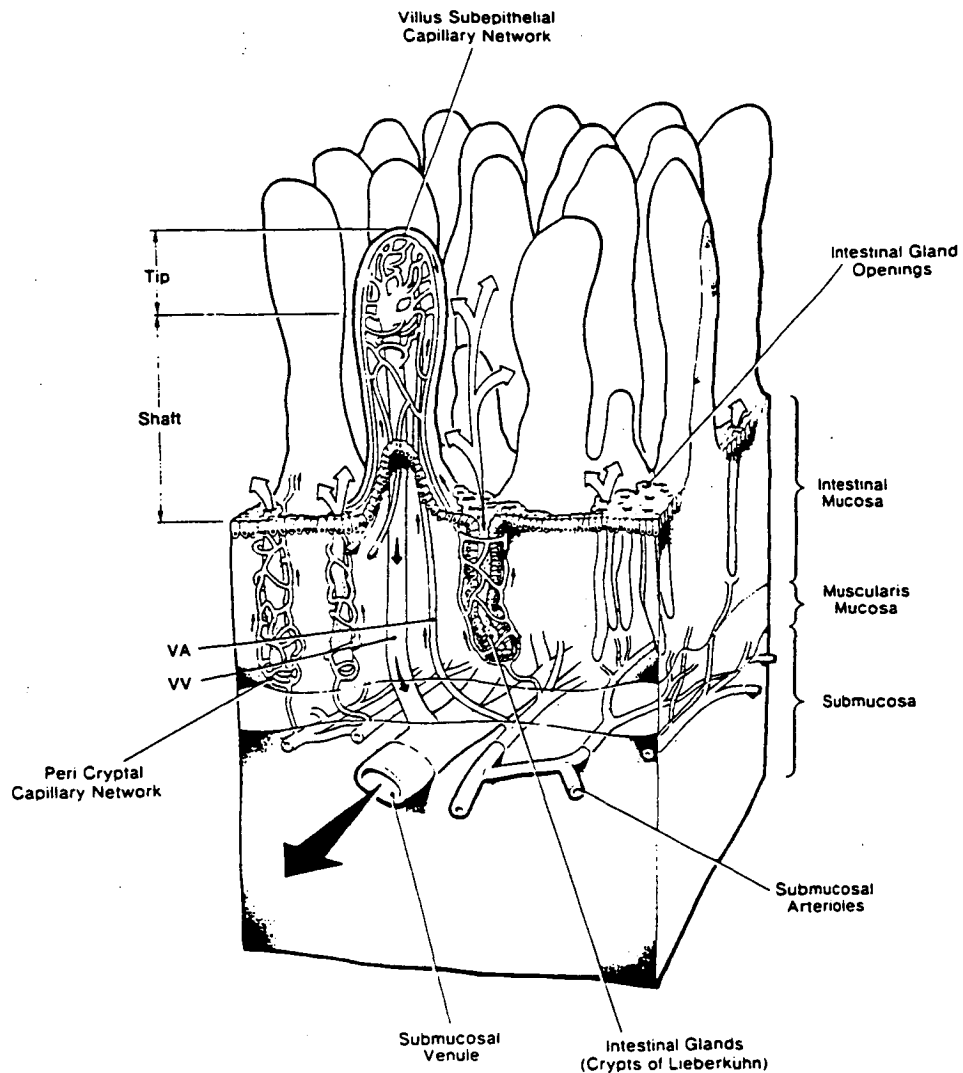
### FURTHER INVESTIGATIONS CONCERNING PC BIOAVAILABILITY

#### 4.1 INTRODUCTION

Physiologically the absorption process is controlled by the nature of the epithelial barrier but is finally dependant on blood and lymph flow away from the intestine. Barr and Riegelman (1970) have defined absorption as the amount of unchanged drug absorbed from the intestinal lumen which appears in the portal circulation or intestinal lymph. Clearly the microcirculation of the intestinal mucosa (Figure 4.1), containing a comparatively high capillary density, is complex and an integral part of the absorption process (Granger *et al.*, 1987). Hence the most revealing studies concerning absorption might be those carried out under experimental conditions which maintain the functionality of the circulatory system and allow sampling from the hepatic portal circulation.

Data from the calcey studies, *in situ* ligated loop and everted sac experiments all suggested that some PC absorption occurred. Additionally, the latter two techniques provided evidence that possible metabolism to citrate might limit the available PC. Hence the results of the previous experiments portrayed two aspects to be considered. Firstly, can transfer of PC across the gut epithelia be demonstrated *in vivo* and secondly, is there significant metabolism of PC, either intralumenally or during passage across the intestine? To answer these and other questions relating to PC absorption, additional experiments which consider the appearance of PC in the portal vein could be informative.

A variety of intestinal perfusion models are reported in the literature (Macdonald, 1989). The system reported by Doluisio *et al.* (1969) is often used



**Figure 4.1** Model of mucosal microcirculation typical of human and rabbit small intestine. VV, villus venule; VA, villus arteriole; solid arrows indicate direction of blood flow; open arrows indicate directions of secretion and absorption of intestinal fluid. (reproduced from Granger *et.al.*, 1987).

for absorption studies. Recently Yorgey *et al.* (1986) and Blanchard *et al.* (1990) have utilised this technique with venous cannulation for studying drug absorption. Techniques for sampling of blood and lymph following oral administration are also well established (Gallo- Torres and Ludorf, 1974; Charman, 1986). The studies reported here use a perfused loop concurrent with portal vein cannulation. Basically the technique is a more elaborate version of the *in situ* loops described by Levine and Pelikan (1961). Two L-shaped cannulae are surgically inserted at either end of a section of intestine and then connected to syringes allowing multiple sampling of the intestinal contents.

Additionally, the potential therapeutic effect of a drug is dependant on the bioavailability and the rate of disappearance or elimination from the systemic circulation. Hence further experiments were devised to gain insight into the nature of the reduced bioavailability of PC following oral administration. These involved measuring the disappearance of PC from the bloodstream following an intravenous bolus of PC. When taken together, the data measuring absorption from the lumen, the appearance of PC in the portal vein and the intravenous disappearance of PC should provide useful information concerning PC absorption, first-pass effects and elimination. This should allow decisions to be made concerning research directions for the development of an appropriate dosage form of PC.

## **4.2 METHODS**

### **4.2.1 Equipment and Reagents**

General equipment used included a liquid scintillation counter (Beckman), water bath heater pump (Techne TE7), temperature controlled oven (Townsen), and centrifuges (Sorvall RC2-B, Eppendorf 5414S, IEC. DPR-

6000). 1,5- $^{14}\text{C}$ - Citrate was obtained from DuPont (NEN research products, Boston, MA.).

#### 4.2.2 Synthesis of radioactive PC

Typically, 1,5- $^{14}\text{C}$ -citrate (250-750  $\mu\text{Ci}$ ) was combined with 10 mg of cold citrate and methylated with diazomethane according to the method of Hudlicky (1980). Solvent was removed by rotary evaporation and the resulting 1,5- $^{14}\text{C}$ -trimethyl citrate stored in a vacuum dessicator prior to use. For synthesis, the 1,5- $^{14}\text{C}$ -trimethyl citrate was added to a small vial containing unlabelled trimethyl (or triethyl) citrate (total 1 mmol) and 3 ml of a benzene/pyridine mixture (0.54 ml pyridine/ 15 ml benzene). O-phenylene phosphorochloridate (1 mmol) dissolved in 3 ml benzene was then added dropwise to the vial and the synthesis and isolation of products then proceeded according to the description in section 2.2.2 with appropriate small scale modifications.

Characterisation of  $^{14}\text{C}$ -PC included TLC on cellulose plates in a solvent system comprising isobutyric acid/ 5.5% ammonia (w/w)/ methanol (60:40:100. v/v) In this system the  $R_f$ 's of citrate and PC are 0.42 and 0.20 respectively. The location of radioactive material was detected using a spark chamber (Birchover Instruments, Herts., England) and photographic detector (polaroid, film type 87).  $^{14}\text{C}$ -PC was also analysed using the method described in section 3.2.2.

#### 4.2.3 Intestinal Cannulation

The cannulation procedure was according to the method of Doluisio *et al.* (1969). Male Hooded Wistar rats weighing 240-260 g were fasted overnight and the following morning anaesthetised with an intraperitoneal injection of sodium pentobarbitol (200  $\mu\text{l}$ , 60 mg/ml diluted 1:1 with saline). The gastrointestinal tract was exposed with a midline abdominal incision. A section

of intestine starting approximately 5 cm posterior to the pyloric sphincter and usually 30 cm in length was isolated. Small incisions were made at the proximal and distal ends of this section and two L-shaped glass cannulae were inserted. The cannulae were secured with silk ligatures and the intestines then returned to the abdominal cavity in a way that left only the cannulae exposed. Glass hypodermic syringes (20 ml) were connected to the cannulae with latex tubing (I.D. 1/32", O.D. 1/8"). The abdominal opening was then covered with a saline moistened (37°C) gauze. A layer of thin plastic sheeting ("Gladwrap") was placed over the gauze. If portal vein cannulation was required, then this surgery was always performed prior to the intestinal procedure.

#### **4.2.4 Portal Vein Cannulation**

A technique similar to that described by Davison (1989), which involved introducing a free floating cannula into the portal vein, was used. The vein was exposed and surrounding adventitious tissue cleared away. Curved forceps were used to hold the vein away from the body wall while two loose ligatures were placed under the vein about 1 cm apart. Correct manipulation resulted in an isolated 1 cm section of vein engorged with blood. This facilitated the cannulation step which involved making a small puncture in the vein with a 19 gauge hypodermic needle followed by rapid insertion of the cannula (PE 50, I.D. 0.50 mm, O.D. 1.00 mm) and release of the ligatures. The end of the cannula was bevelled to facilitate entry into the vein and inserted to a depth of 1.0 cm towards the liver. The cannula was secured by suturing in two places to the abdominal wall.

Clotting was prevented by first filling the cannula with heparin (David Bull Labs., Aust., porcine sodium heparin, 1000 units / ml) and inserting a small plug in the free end. Before sampling, the heparin was withdrawn together with

a small volume of blood and a fresh syringe was connected.

#### **4.2.5 Vena cava cannulation and perfusion**

Cannulation was according to Kaufman (1980). The vena cava was exposed by moving the abdominal contents to one side and dissecting away surrounding fat and connective tissue. The cannulation step was similar to that described above for the portal vein cannulation. Silk ligatures were positioned in place 1.0 cm apart. The vein was punctured 1.0 cm above the ileac bifurcation and a heparin filled cannula (PE 50) with a bevelled end inserted to a depth of 1.0 cm.

For perfusions, a solution containing  $1,5\text{-}^{14}\text{C-PC}$  ( $195\ \mu\text{l}$ ) was introduced into the vena cava as a bolus containing 5 mg of PC. The specific activity of the radioactive preparation was  $1.738 \times 10^6$  cpm/mg PC.

#### **4.2.6 Intestinal Perfusion**

Isotonic saline was slowly perfused for a period of 5 min, followed by Krebs - Henseleit (Dawson, 1986, p446) buffer for a further 15 min. The experiment was then commenced by introducing Krebs - Henseleit buffer containing an appropriate concentration of PC. Solutions were maintained at  $37^{\circ}\text{C}$  and Krebs - Henseleit buffer was equilibrated with 5%  $\text{CO}_2$  / 95%  $\text{O}_2$  prior to perfusion. Sampling was conducted at time intervals during the experiment by expelling the perfusate into one of the syringes using air pressure derived from the other syringe. This procedure resulted in adequate mixing of the perfusate before sampling and a short time interval between expulsion and returning the perfusate to the intestine. Manipulation and disconnection of the syringes was made possible by pinching the tubing which joined the cannula to the syringe. Maintenance of body temperature was facilitated by using a heating

lamp and heating pad. Perfusate volumes were recorded at each sampling as the syringes were calibrated for volume determination.

Perfusions were initiated by introducing the perfusion medium and immediately taking an aliquot of the perfusate. This was defined as the zero time sample. The perfusion arrangement is shown in Figure 4.2.

#### **4.2.7 Chromatography**

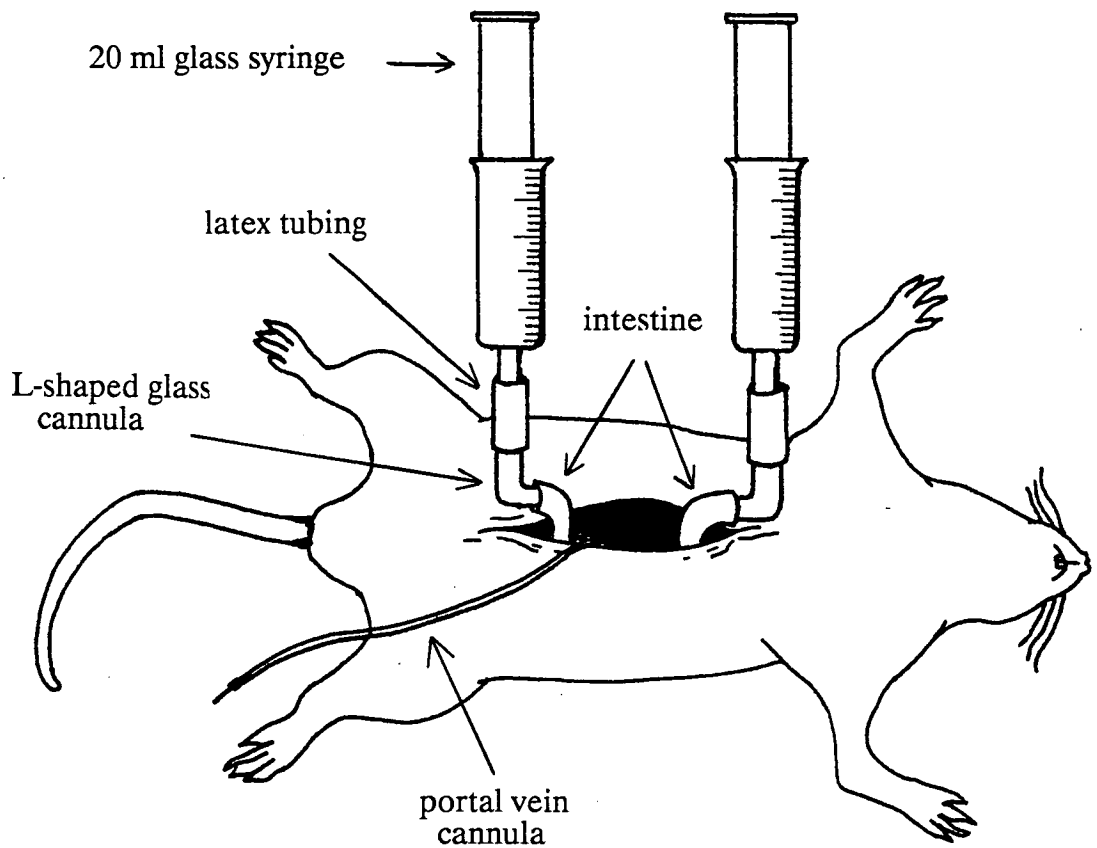
##### **4.2.7.1 Treatment of Blood Samples**

The procedure was conducted at 4°C and prior to use, reagents were kept on ice. Extraction involved adding perchloric acid (1 M, 600 µl) to whole blood (300 µl) and mixing vigorously. After 2 min on ice, the acid extract was centrifuged (Eppendorf, 2 min, 8000g, 4°C) and the supernatant transferred to another tube containing one drop of phenol red indicator (BDH chemicals, 0.02%). Potassium carbonate (2 M) was added dropwise until neutrality was indicated. The resulting mixture was then centrifuged (Eppendorf, 2 min, 8000g, 4°C) and the supernatants lyophilised. Larger volumes were treated in a similar manner with appropriate volume changes and centrifugation in a Sorvall centrifuge (RC2B, 10,000g, 4°C).

##### **4.2.7.2 Chromatographic analysis of Extracts**

Lyophilised samples were reconstituted in a minimum volume and applied to Whatman No. 1 chromatography paper as a thin band 10 cm long. Several applications were required allowing the paper to dry between applications so that maximum loading was achieved. The paper was then developed by descending chromatography (2.5 h) in a solvent system comprising





**Figure 4.2** Perfusion system used for measuring intestinal absorption. (based on Doluisio *et. al.*, 1969)

n-propanol/ water/ glacial acetic acid (4:1:1. v/v)

After the designated running time the chromatograms were cut into strips (0.5-1.0 cm) parallel to the application band and then subsequently cut into smaller pieces and packed into 2.5 ml plastic syringes. The contents on the paper were extracted twice with 1 M HCl (0.5 ml). During each extraction the packed strips were allowed to soak in HCl for 5 min before centrifugation (1000 g, 5 min) directly into plastic scintillation vials for counting. A rubber grommet allowed the syringe to fit tightly over the vial during centrifugation.

#### **4.2.8 Digestion and counting of Blood Samples**

Blood samples were processed as described by Williams (1981). The procedure ensured efficient recovery of the radiolabelled compounds with digestion achieved by the addition of an oxidative mixture comprising perchloric acid (70% w/v, 200  $\mu$ l) and hydrogen peroxide (30% w/w, 400  $\mu$ l) in a glass counting vial. The vials, fitted with acid resistant caps, were heated (50-60°C) until the solutions turned colourless. This process usually took 1-2 h after which the vials were allowed to cool to room temperature before the addition of scintillant (10 ml) and counting. The scintillant (Fricke, 1975) was an acid resistant cocktail composed of 3.0 g PPO (2,5-diphenyloxazole), 257 ml Triton X-100, 106 ml Ethanol, 37 ml Ethylene glycol and 600 ml Xylene.

### **4.3 RESULTS**

#### **4.3.1 Intestinal perfusion of PC**

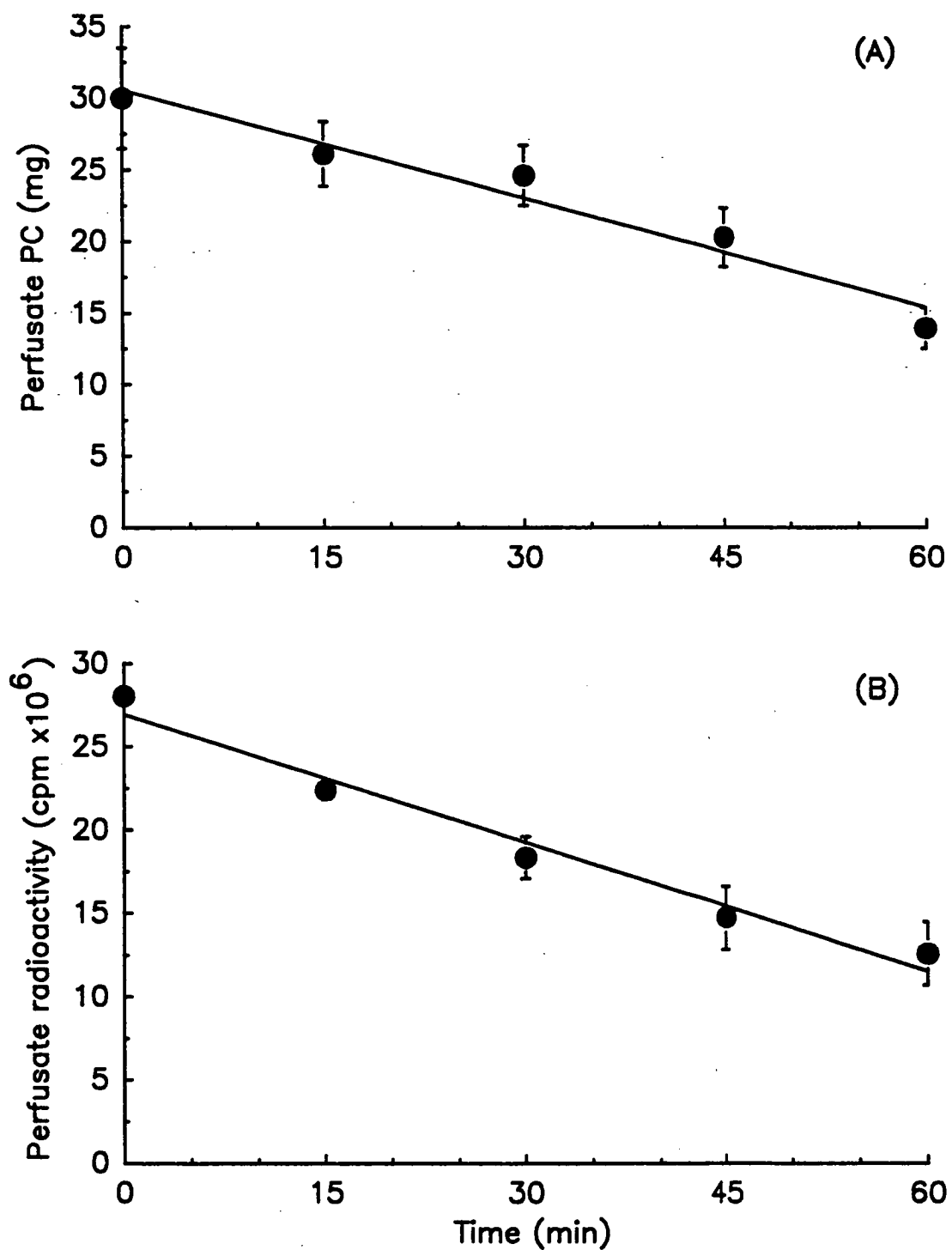
Absorption of 1,5-<sup>14</sup>C-PC was measured following *in vivo* perfusion with 5 mM PC. Figure 4.3 (A) and (B) show the disappearance of PC from the lumen as determined by enzymatic assay for PC (A) and the disappearance of total

radioactivity (B). Figure 4.3 (A) indicates an absorption rate of 16 mg (36  $\mu\text{mol}$ )/ 30 cm intestine/ h. This corresponded to 53% of the PC present at time zero. Figure 4.3 (B) indicates that 55 % of the total radioactivity present at time zero disappeared in 1 h. Additionally, the uptake of radioactivity by portal blood during four individual experiments was measured (Figure 4.4). In all profiles, radioactivity increased with time and in one experiment a plateau of absorption was reached. The presence of PC in portal blood was determined by chromatographic analysis and is shown in Figure 4.5. The data revealed limited absorption at 15 and 30 min but the 45 and 60 min samples did indicate the presence of PC in portal blood. At each time point the presence of radioactive citrate was also apparent from the chromatographic profiles. Radioactive material which did not correspond to standard PC or citrate, was also noted in the profiles.

The possibility of PC absorption being related to a tricarboxylic acid carrier was briefly explored by conducting some experiments which included a transport inhibitor. Figure 4.6 shows that the presence of 2.5 mM tricarballylate, a tricarboxylic acid transport inhibitor (Browne *et al.*, 1978), in the perfusion medium did not affect the uptake of PC. However there was an indication of increased citrate accumulation in the intestinal lumen.

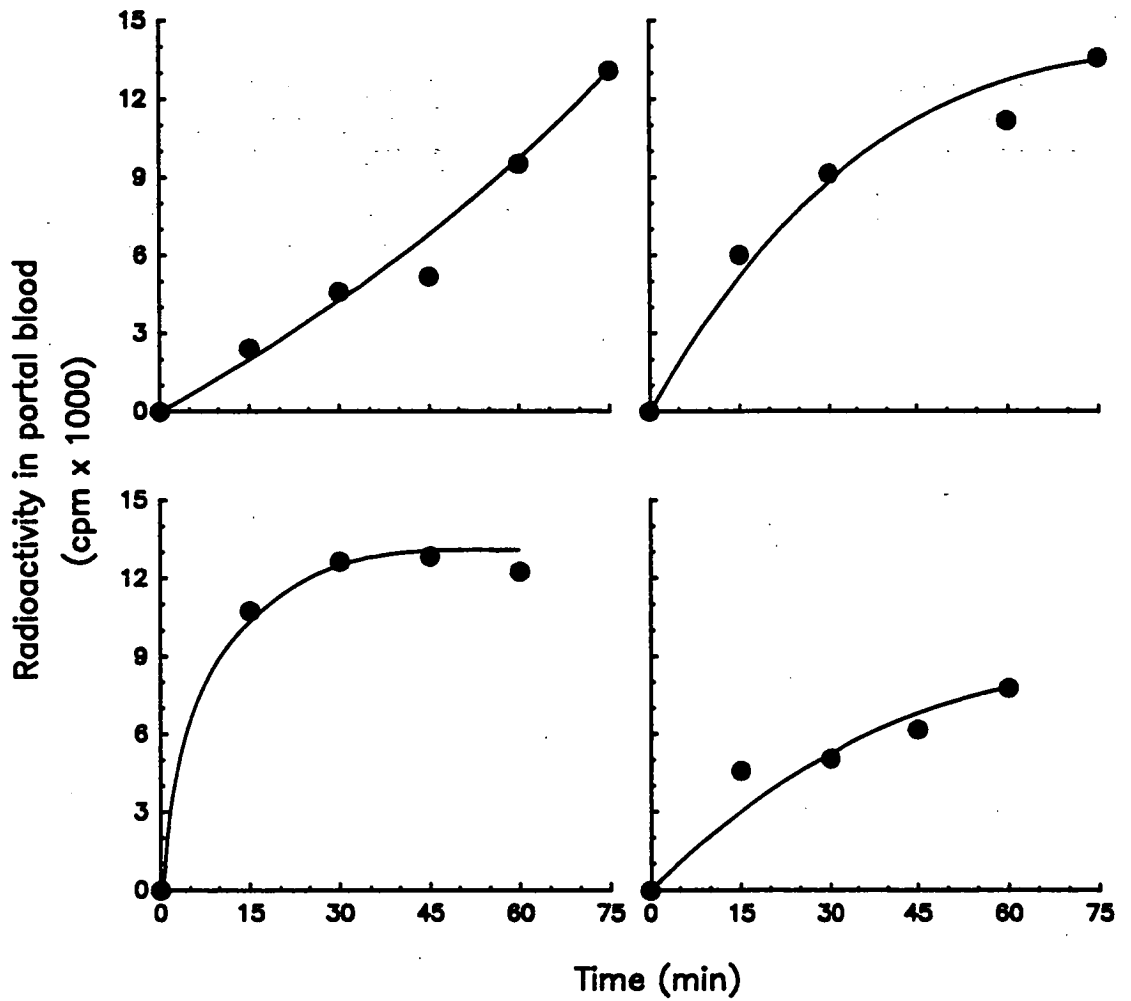
#### 4.3.2 Disappearance of PC following I.V. administration

Figure 4.7 shows the non-linear disappearance of 1,5- $^{14}\text{C}$ -PC from the circulation following an intravenous bolus. At 1 min the radioactivity in blood was 12,099 cpm/200  $\mu\text{l}$ . On the basis of an average total blood volume of the rat being 64.1 ml/kg (Altman and Dittmer, 1974) this corresponded to 11.1% of the total counts administered. Chromatographic analysis (Figure 4.8) indicated that



**Figure 4.3** Disappearance of PC from intestinal perfusate.

A: Data measured as total PC by enzymatic assay. B: Data measured as perfusate radioactivity. Data represents mean  $\pm$  SEM. (n=4)



**Figure 4.4** The appearance of radioactivity in portal blood during intestinal perfusion with 1,5-<sup>14</sup>C-PC. The data shows the results of four individual experiments.

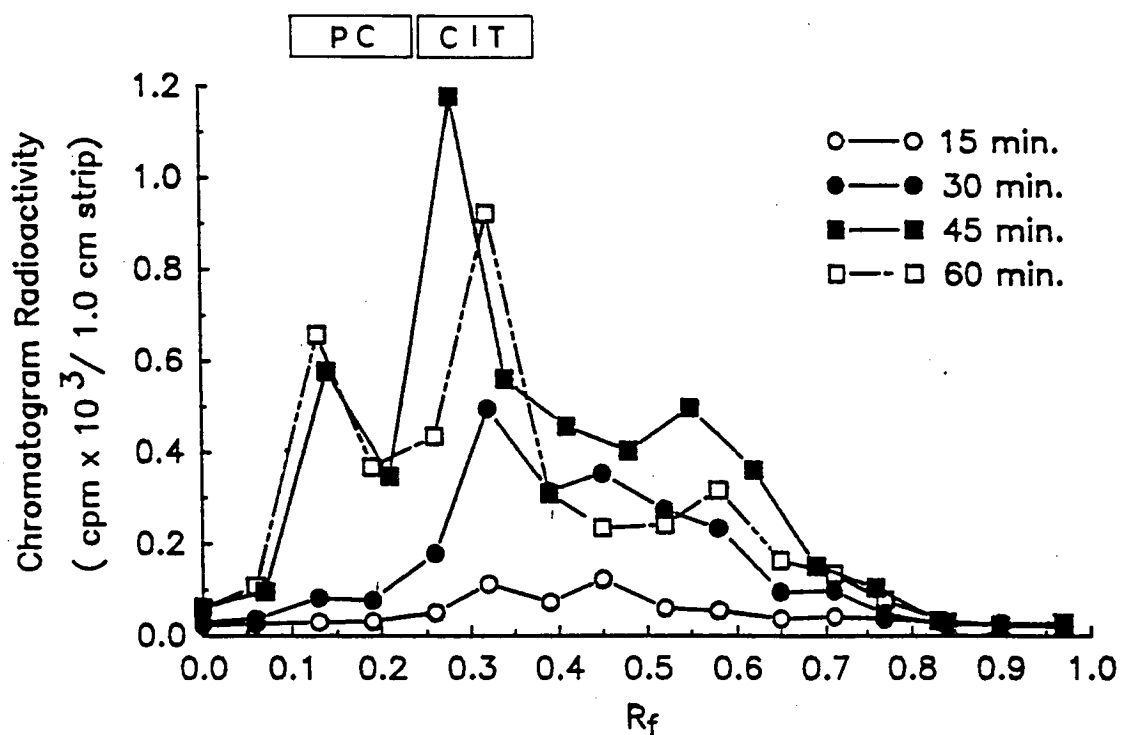
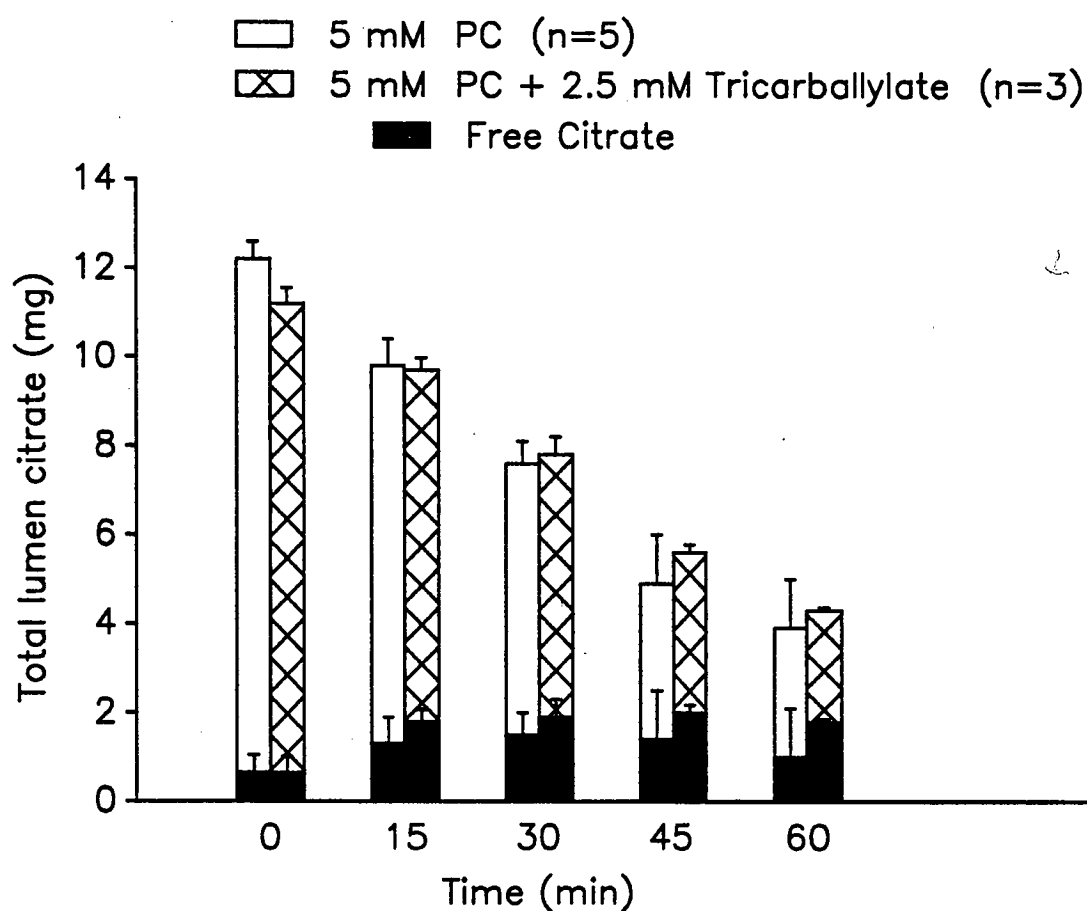
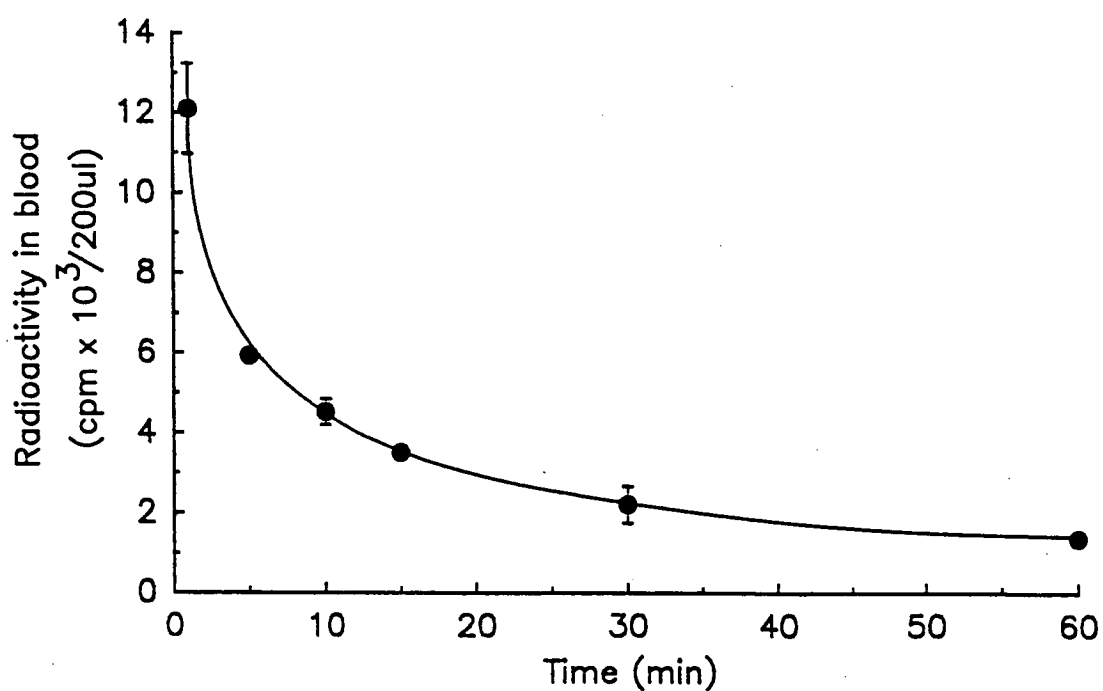


Figure 4.5 The pattern of radioactivity appearance in portal blood during intestinal perfusions. Protein free extracts were chromatographed. Chromatograms were divided into 1.0 cm strips for determination of radioactivity. The presented data is from a single experiment. However the studies were repeated on three other occasions.

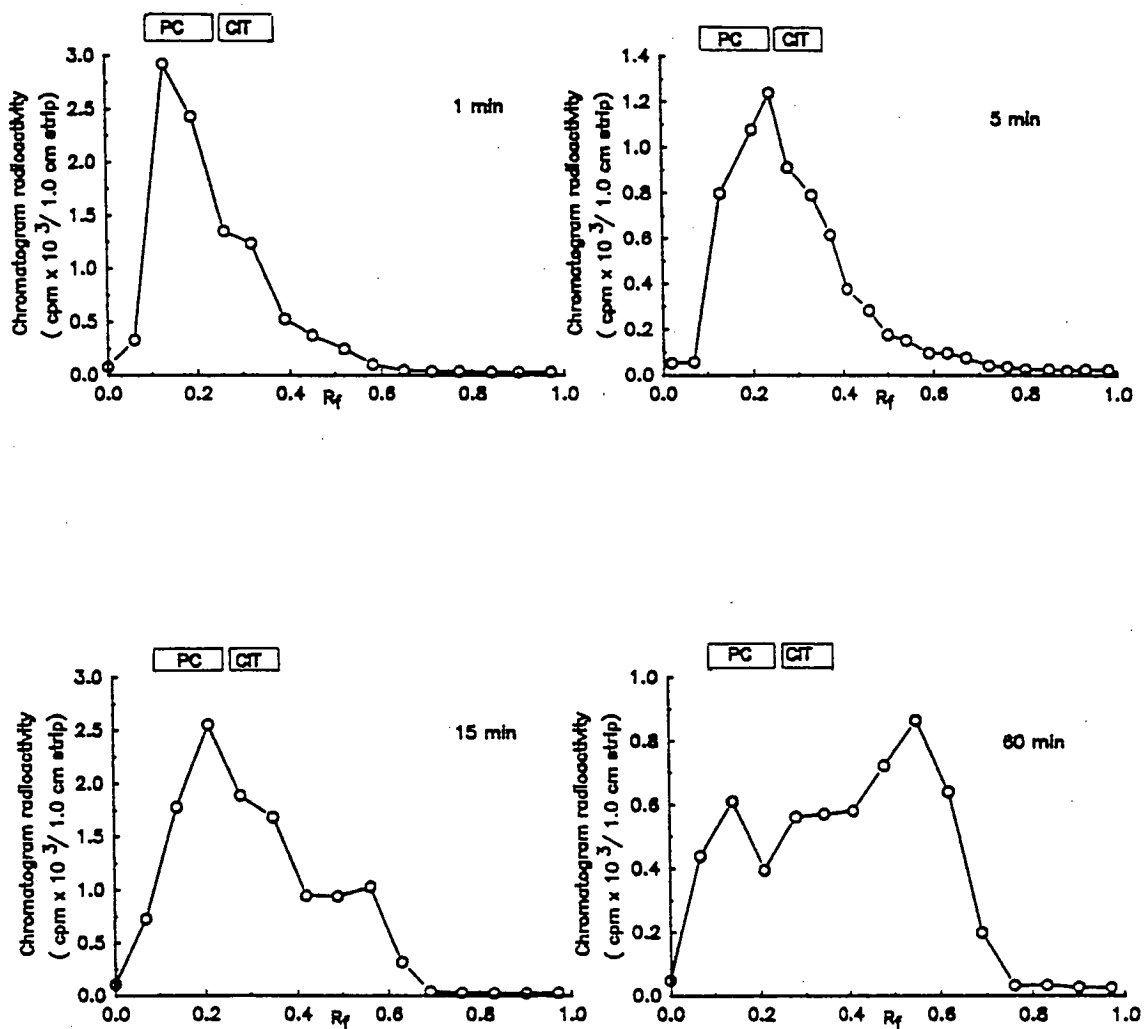


**Figure 4.6** The effect of tricarballylate on the uptake of PC from the intestinal lumen. Total citrate as determined by *in vitro* assay of recovered samples (section 3.2.2) refers to hydrolysable PC plus free citrate. Data represents mean  $\pm$  SD.



**Figure 4.7** Disappearance of radioactivity from the blood stream following intravenous administration of  $1,5\text{-}^{14}\text{C-PC}$ . Data represents mean  $\pm$  SEM. (n=3)





**Figure 4.8** Chromatographic analysis for radioactivity in systemic blood samples following an intravenous bolus of 1,5-<sup>14</sup>C-PC. The data was obtained as for figure 4.5.

the radioactivity was present mostly as PC. At 5 min radioactivity was reduced to 5941 cpm/200  $\mu$ l whilst at 15 and 60 min, the radioactivity was 3505 and 1372 cpm/200  $\mu$ l respectively. The chromatographic profiles for these time intervals indicated that a decreasing proportion of the counts was due to PC.

#### 4.4 DISCUSSION

Radiolabelled PC was used to demonstrate transport across the gastrointestinal tract. Absorption of PC from the lumen was clearly established. Disappearance of radioactivity matched the uptake of PC, as was determined by assay for PC. This data is in line with the information obtained in the previous chapter. Further information regarding absorption was also obtained following analysis of portal blood samples. The data provided conclusive evidence that PC was appearing in the portal vein following lumen perfusion. This was anticipated as previously, partial inhibition of calcergy was observed following oral delivery of PC. Chromatographic data indicated that radiolabelled citrate also appeared in the portal vein which was suggestive of the possibility of first-pass metabolism of PC in intestinal tissue.

However, although the chromatographic data suggested that more citrate was appearing in the portal circulation than PC, if citrate was absorbed at a faster rate than PC this pattern might result even if metabolism was not a limiting factor. Citrate is believed to be absorbed by a specific mechanism (Browne *et al.*, 1978; Wolfram *et al.*, 1990). Therefore if PC is absorbed by a restricted passive process, then it is possible that the citrate which appeared in the portal vein was a higher proportion of the total radiolabelled citrate than was the case for PC. In support of this, the appearance of PC in portal blood was not apparent until at least 45 min into the perfusion. In addition, the

chromatographic profiles of portal blood indicated that citrate appeared at an earlier time-point than PC during the perfusion.

Having established that PC appeared in the portal vein and that some gut metabolism had occurred, some future consideration might also need to be given to the role of the liver in any discussion of first pass metabolism. Although results obtained by Williams (1981) showed that the liver did not accumulate large amounts of radioactivity following intravenous injection of labelled PC, nevertheless, this organ may still influence the ultimate systemic concentrations reached following oral delivery of PC.

Also there is a need to further quantitate the actual rate of PC absorption and metabolism. Techniques which may aid this process include using a total mesenteric collection or complete vascular perfusion (Windmueller and Spaeth, 1984). Such techniques however could be applied best if a highly sensitive assay for PC was available. The current absence of a very high specific activity  $^{14}\text{C}$ -PC preparation or an ultra sensitive assay for unlabelled PC in blood, limited the overall detection and quantitation of PC. This was further complicated by the rapid disappearance of PC from the systemic circulation which meant that concentrations of radioactive PC and metabolites in blood were reduced to levels which were at the detection limits of the analytical system used.

Another aspect to PC absorption was the investigation of the transport mechanism with regard to localisation within the intestine. Although this area does not fall completely within the brief of the current study, some preliminary experiments were instigated and it was found that PC disappeared from the intestinal lumen throughout the length of the intestine. Further studies which

measure the appearance of PC in the portal vein following perfusion of various intestinal segments could also be helpful especially in regard to metabolic events taking place. Enzyme activity is known to vary considerably throughout the length of the intestine (Weiser *et al.*, 1986).

Preliminary studies concerning the mechanism of PC uptake were initiated using tricarballylate, an inhibitor of tricarboxylic acid transport. Generally, the data obtained (this chapter and everted sacs) indicated that PC absorption was slow and possibly via a passive process as opposed to an active or facilitated transport mechanism. That tricarballylate did not alter the absorption profile of PC may also be suggestive that uptake is not via a known active process. Marginally increased levels of citrate within the lumen were apparent indicating that some inhibition of citrate uptake may have occurred.

The disappearance of PC from the systemic circulation following an intravenous bolus is relevant to the issue of gut absorption. The data indicates that greater than 90% of injected PC disappeared from the circulation within 1 min. This rapid rate of disappearance of PC from the blood coupled with its slow absorption from the intestine might combine to render PC less effective orally than by intraperitoneal injection. Perhaps then PC, following oral administration, does not reach threshold levels necessary for the effective prevention of calcergy formation observed following intraperitoneal administration.

Chromatographic studies showed that the proportion of radioactivity attributable to PC, following the administration of an intravenous bolus, decreased in time with citrate appearing as the main metabolite. This indicated that some PC does undergo metabolism after entering the systemic circulation.

It is not clear whether the appearance of radioactive metabolites is due to enzymic activity in blood or follows the release from tissue after uptake and breakdown of PC.

Overall, the data obtained in this study so far has indicated that PC was absorbed and was active following oral delivery of the compound. However the need for improved forms of the compound for oral administration is clear. Prodrugs have been used for many drug compounds when carboxylic acid functional groups complicate the absorption process (Waller and George, 1989) making it a possible approach for increasing the systemic availability of PC following oral delivery.

## CHAPTER 5

### SYNTHESIS AND ANTICALCIFYING POTENTIAL OF PC ESTERS

#### 5.1 INTRODUCTION

The poor bioavailability of PC following oral delivery would appear to be a property of its physical characteristics. Whilst the high charge to size ratio and stereochemistry of the molecule is clearly essential to its inhibitory power it is obviously not conducive to efficient uptake of the compound from the gut. The data suggests that to improve its transport efficiency across membranes, a strategy is needed which can deliver PC intact to the appropriate tissue following oral delivery.

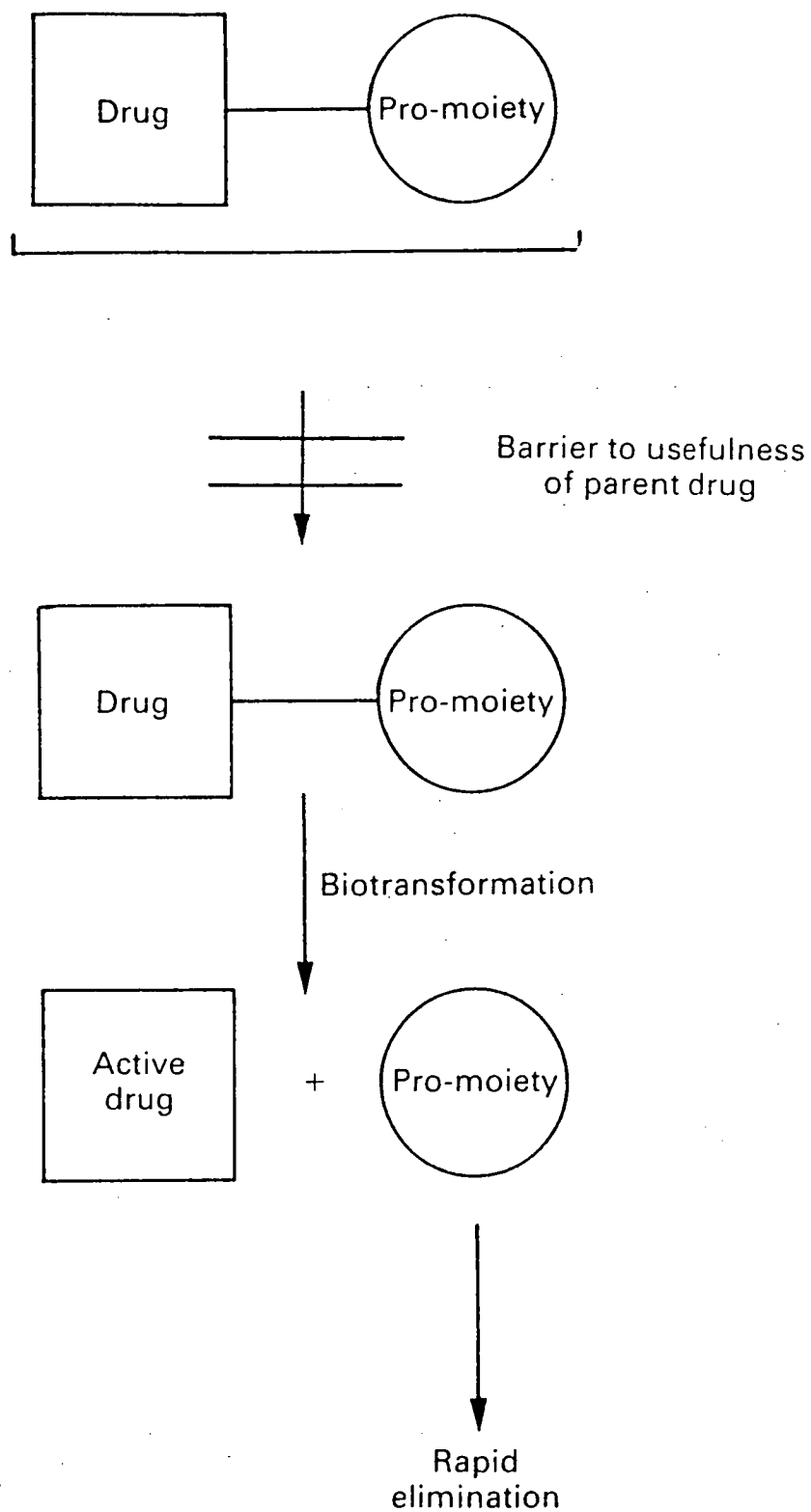
Generally, the bioavailability of a drug can be improved by physical, biological or chemical means (Sinkula and Yalkowsky, 1975). The physical approach primarily involves manipulation of the dosage form to achieve a maximum response. The biological approach involves variation in the route of administration. However this strategy is often limited due to the inconvenience of many administration routes. Clinically the oral route is preferred and problems concerning absorption may need to be directly overcome. For this reason the chemical approach is sometimes the most useful avenue to improved bioavailability.

Chemically, two options are available: (i) Synthesise a chemical analog which would be expected to differ from the original in efficacy and a range of other pharmaceutical parameters (Sinkula, 1982). However the essential bioactivity of the parent compound would have to be retained. (ii) Develop a

prodrug by chemically altering a known drug to form an inert molecule which following administration would undergo biotransformation to yield the active compound and an inert pro-moiety (Figure 5.1). A range of derivatisations have been used for this purpose ranging from standard chemical linkages including amides, esters and phosphates (Notari, 1981) to more novel approaches such as the synthesis of methylthiomethyl derivatives of carboxylic acids (Bodor, 1982). The type of chemical modification actually required needs to be considered in light of reactive functional groups on the parent molecule and on the availability of *in vivo* systems for bioactivation of the prodrug. In respect to the latter, a range of enzymes have been used, including esterases, amidases, phosphatases and reductases (Sinkula and Yalkowsky, 1975).

Prodrugs have been used to aid a range of pharmaceutical problems including improvement of taste, decreasing dangerous side effects, decreasing gastro-intestinal irritation, increasing solubility and obtaining site specificity (Notari, 1981). Another factor commonly overcome using the prodrug approach is the oral absorption of water soluble and highly polar compounds. Derivatisation with lipophilic groups has been used in many instances to overcome their limited absorption. More specifically, esterification with a hydrophobic moiety will often improve the absorption of a drug containing a carboxylate functional group (Sinkula and Yalkowsky, 1975), two notable examples being ampicillin (Clayton *et al.*, 1976) and carbenicillin (Clayton *et al.*, 1975).

Therefore the development of a prodrug form of PC was envisaged as a tool to increase its transport across the gut. Accordingly, the use of alkyl esters of PC was considered as (1) the proposed compounds should have increased



**Figure 5.1** The prodrug concept (reproduced from Waller and George, 1989)



lipophilicity and (2) such compounds should be converted back to active PC as a range of enzymes able to hydrolyse short to medium chain aliphatic ester derivatives exist (Sinkula and Yalkowsky, 1975). These include cholinesterases, carboxylesterases, acetyl esterases and lipases. Carboxylesterases (Krisch, 1971), which could be considered the most likely enzyme group to hydrolyse the ester bonds of PC alkyl esters, are commonly found in high activity in liver, duodenum, kidney and brain (Waller and George, 1989). The possibility that esterification might reduce any first-pass effects through steric inhibition of enzymic or chemical hydrolysis of PC might also be an advantage.

The availability of phosphotriethyl citrate (PTEC) as an impure intermediate in the synthesis of PC (section 2.2.2) provides a starting point regarding synthetic possibilities. If a suitable synthetic strategy could be developed then the potential for a prodrug compound of PC to undergo hydrolysis and release active PC into the systemic circulation would need to be tested. In this respect it was envisaged that the previously established calcergy model (section 2.2.2) would again be suitable.

## **5.2 EXPERIMENTAL**

### **5.2.1 Synthesis of phosphotriethyl citrate**

The reaction mixture obtained following the first step in the synthesis of PC (section 2.2.2) was dissolved in  $\text{CH}_2\text{Cl}_2$  and applied to a silica column (I.D. 1.4 cm x 25 cm) pre-equilibrated with  $\text{CH}_2\text{Cl}_2$ . The column was initially eluted with  $\text{CH}_2\text{Cl}_2$  (150 ml) after which the eluting solvent was changed to 2% MeOH in  $\text{CH}_2\text{Cl}_2$ . The first 50 ml of eluant was discarded and thereafter

fractions (5 ml) were collected. The progress of separation was monitored by TLC. Fractions which contained the coupled product (triethylcitrate coupled to o-phenylene phosphochloridate) were combined and hydrogenated (section 2.2.2). Following hydrogenation the mixture was neutralised (NaOH, 1M), rotary evaporated, extracted into  $\text{CH}_2\text{Cl}_2$  and rotary evaporated again resulting in a white crystalline material with a typical ester aroma.

### 5.2.2 Partial synthesis of phosphotrioctyl citrate

The planned strategy for the synthesis of phosphotrioctyl citrate required the initial synthesis of trioctyl citrate, subsequent phosphorylation, followed by isolation and purification. Accordingly trioctylcitrate was synthesised from citrate as described by Pelister and Breusch (1976) for the esterification of propene-1,2,3-tricarboxylic acids. Citrate (25 mmol) and octanol (82.5 mmol) were added to a 100 ml round bottom flask together with 481 mg of p-toluene sulphonic acid (5% of citrate weight) in 50 ml of toluene. A calibrated trap for water collection and a reflux condensor was fitted and the reaction proceeded under reflux for 2-3 h or until the evolution of water discontinued. The mixture was washed with water (3x50 ml), 5%  $\text{KHCO}_3$  (2x50 ml) and finally with 50 ml water before drying over sodium sulphate. The drying agent was removed by filtration and the toluene by rotary evaporation. Trioctyl citrate was crystallised at  $-20^\circ\text{C}$  from a minimum volume of toluene, washed with ethanol and recrystallised from ethanol (x2) at  $-20^\circ\text{C}$ .

Trials were conducted to investigate the possibility of synthesising phosphotrioctyl citrate by phosphorylation with o-phenylene phosphochloridate and hydrogenation using identical techniques to those described for the synthesis of phosphotriethyl citrate (section 5.2.1).

### 5.2.3 Monoester synthesis

#### 5.2.3.1 Preparation of polymer linked $\text{AlCl}_3$

Polymer linked  $\text{AlCl}_3$  was prepared according to the method of Neckers *et al.* (1972). Accordingly, 15.5 g of polystyrene-divinyl-benzenecopolymer beads (3.5%, 50-100 mesh, Sigma) was combined with carbon disulfide (225 ml) and aluminium chloride (3.75 g, anhydrous) in a 3-neck 1 l flask equipped with a mechanical stirrer, condenser and dropping funnel. The mixture was stirred under reflux for 40 min, then cooled before the addition of water (200 ml). The beads were filtered and washed successively with 1 l of water and 150 ml each of ether, acetone, hot isopropanol and ether.

#### 5.2.3.2 Synthetic trials using PC-bead combinations

Various combinations of polymer beads, PC and alcohols were used in the exploration of monoester synthesis. The following description represents a typical preparation: Free PC was prepared by removing sodium from the usually available sodium salt. Accordingly, PC (45 mg) was dissolved in water (500  $\mu\text{l}$ ) and applied to a small column (15x8 mm) of AG.50W-X8 in the  $\text{H}^+$  form. The PC was collected in the second 500  $\mu\text{l}$  of eluate and n-butanol (740  $\mu\text{l}$ ), polymer- $\text{AlCl}_3$  beads (100 mg) and benzene (500  $\mu\text{l}$ ) were then added. The mixture was heated at 80°C with shaking for 3 h before filtering, evaporating to dryness under a stream of  $\text{N}_2$ . Trials varied and involved using butanol, propanol and ethanol under conditions which varied in temperature from 50- 80°C.

#### 5.2.3.3 Monoesters from hydrolysis of PTEC

PTEC preparations were subjected to various hydrolytic conditions involving differences in temperature (0-80°C) and base content after which the solutions were neutralised (HCl, 1 M) and freeze dried. Primarily two types of

alkali, sodium and strontium hydroxide, were used and the quantities varied from a one third molar equivalent of PTEC to a large excess. Sometimes the method of Meyer *et al.* (1959) was used.

#### **5.2.4 General assays**

PC, citrate and phosphate analyses were performed by previously described methods (section 3.2.3). Ethanol was determined according to the method described by Bernt and Gutmann (1974).

#### **5.2.5 Chromatographic procedures**

##### **5.2.5.1 Gas chromatography (GC)**

A Hewlett Packard (model 5890A) gas chromatograph fitted with a poropak Q column (80-100 mesh, Waters assoc. inc. Mass. USA), and a flame ionisation detector linked to a Shimadzu printer/analyser (model C-R3A, Kyoto, Japan) was used. Samples were dissolved in CS<sub>2</sub> and injected (5-10 ul). The operating conditions were: injection port 180°C; oven temperature 250°C; detection temperature 200°C and the carrier gas was nitrogen.

##### **5.2.5.2 Thin layer chromatography (TLC)**

Fractions obtained in the synthesis of PTEC were analysed by TLC, which was performed on either silica gel or cellulose coated plates. Aluminium sheets precoated with silica gel 60 F254 (Merck, 0.2 mm thickness) were used. For cellulose chromatography, glass plates were coated with Sigmacell (type 20) to a thickness of 0.25 mm, dried and stored prior to use. The solvent system for silica TLC was CHCl<sub>3</sub>/MeOH (80:20) whereas for chromatography on the cellulose plates the solvent systems were either isobutyric acid: 5.5% ammonia: methanol (66:40:100) or eucalyptol: n-propanol: formate: water (50:50:20:10).

For the analysis of products obtained in the synthesis of phosphotrioctyl citrate, several solvent systems were trialled. Ultimately, the two most satisfactory were (a) isobutanol: isopropanol: ethanol: water: 25% ammonia (25:20:34:0.7:10) and (b) chloroform: ethanol: water: triethylamine (30:34:8:35)

#### **5.2.6 High voltage electrophoresis (HVE)**

Some samples were identified following separation by paper HVE (Equipment purchased from Paton Industries Pty. Ltd., Beaumont, S. Aust.). The electrophoresis was performed on Whatman 3MM paper at 2000 v for 1.5 h using 0.05 M triethanolamine bicarbonate buffer, pH 7.5.

Samples which had been subjected to hydrolysis (section 5.2.3.3) were subsequently prepared for analysis by HVE. The hydrolysates were vacuum dried and the resulting lyophilisates were dissolved in 1 ml of water before application to a cation exchange column (AG.50W-X8, H<sup>+</sup> form) (I.D. 0.45 cm x 4 cm). The column was eluted with 1 ml of water and the eluants were combined. Samples (5 ul) were applied to paper for analysis.

#### **5.2.7 Visualisation techniques**

##### **5.2.7.1 Organic acids and phosphates**

Organic acids and phosphates were detected according to the method of Firmin and Gray (1974) which required the following reagents:

Solution A: 1.1% Ammonium thiocyanate in acetone

Solution B: 0.03% FeCl<sub>3</sub>.6H<sub>2</sub>O in acetone

Electrophoretograms were dipped briefly in solution A and air dried for 5 min at room temperature before dipping in solution B and again air drying. Organic acids and phosphates showed as bleached white stains on a reddish brown background.

#### 5.2.7.2 Phosphate specific stains

Two general phosphate detection stains were used:

(a) Molybdenum blue visualisation reagent (Maile *et al.*, 1977).

Solution 1: 1 l of 25 N  $\text{H}_2\text{SO}_4$  and 40.11 g of  $\text{MoO}_3$  were mixed and gently boiled until the  $\text{MoO}_3$  was dissolved (3-4 h). The solution was allowed to cool at room temperature overnight.

Solution 2: Powdered molybdenum (1.78 g) was dissolved in 500 ml of solution 1 by boiling gently for 15 min before cooling and decanting to separate any remaining residue.

Working reagent: Equal volumes of solutions 1 and 2 were added to 4.5 vol. of water to form a green solution which was sprayed onto plates and paper electrophoretograms. Phosphate containing compounds were visualised as blue spots.

(b) Ammonium Molybdate-Perchlorate (Dawson *et al.*, 1986)

The reagent which comprised 60% w/v perchloric acid: M-HCl: 4% ammonium molybdate: water (5:10:25:60) was sprayed onto chromatography plates which were then heated at  $85^\circ\text{C}$  for 1 min. Free phosphate was visualised as a yellow-green spot and unhydrolysed phosphoric esters as blue spots.

#### 5.2.7.3 Detection of octyl containing compounds - Iodine vapour

Chromatograms were developed by placing in a tank containing iodine crystals. Iodine absorbing compounds were revealed as brown to yellow spots on a faint yellow background.

#### **5.2.7.4 Ultra-violet visualisation of chromatograms**

Chromatograms were viewed under an ultra-violet lamp (254 nm). Ultra-violet absorbing compounds appeared as darkened regions on the chromatogram.

#### **5.2.8 Calcergy studies with PTEC**

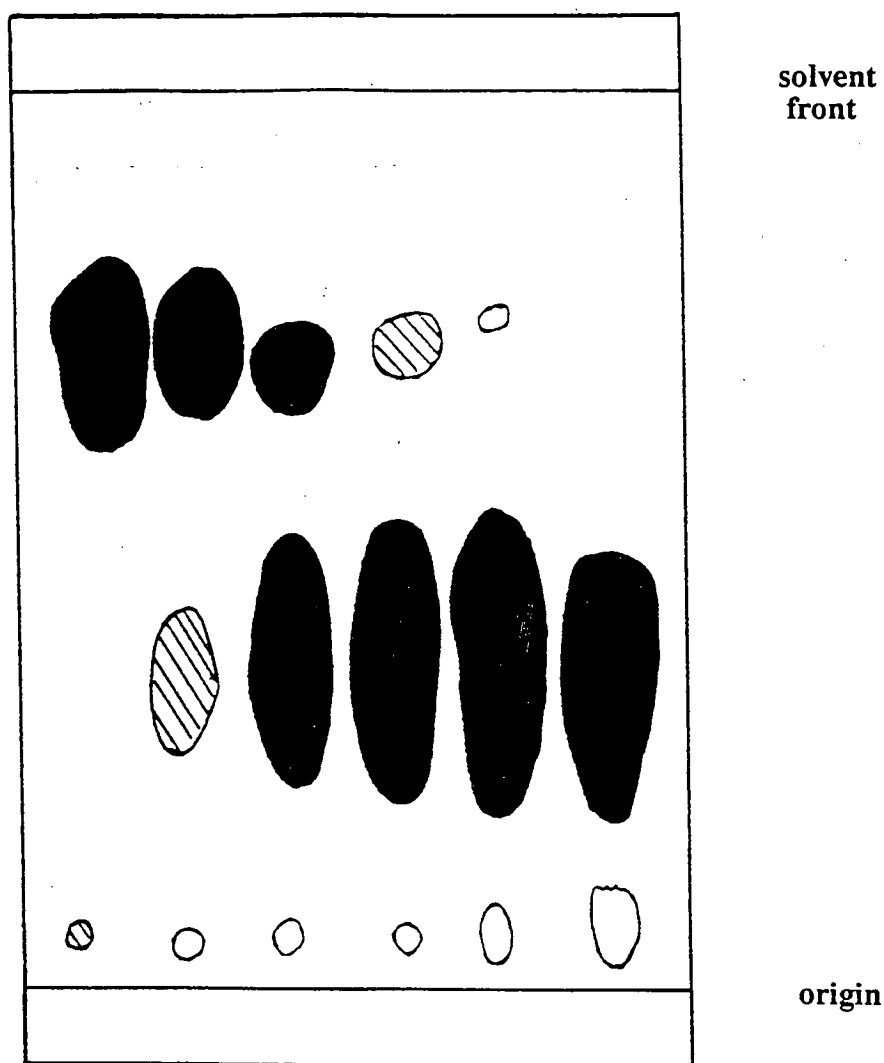
The inhibitory power of PTEC was tested using the calcergy model described in chapter 2. The compound was administered as a solution in water, by gavage, intraperitoneal or subcutaneous injection at a dose level of 50 mg/kg/day.

### **5.3 RESULTS AND DISCUSSION**

#### **5.3.1 Synthesis, isolation and characterisation of PTEC.**

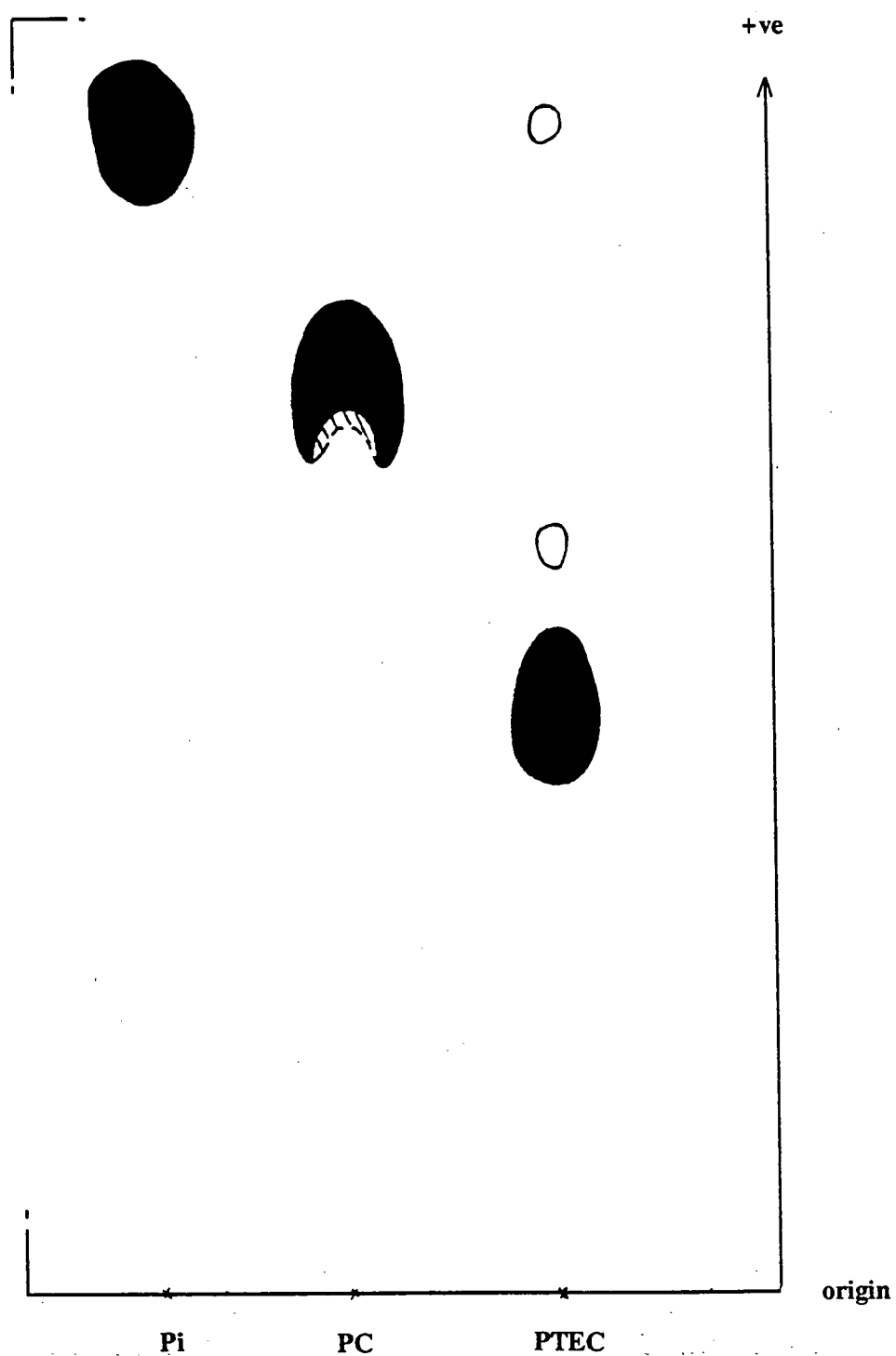
Of the various chromatographic media which were trialled in order to effect the separation of PTEC, preparative silica column chromatography prior to hydrogenation was found to give the best separation. Figure 5.2 shows a typical TLC analysis of fractions obtained during this procedure. The middle spot ( $R_f=0.4$ ), identified as containing PTEC, increased in size and intensity as compared to the top spot ( $R_f=0.7$ ).

PTEC was characterised by HVE,  $^1\text{H}$ -NMR, and analysis for free and hydrolysable phosphate. Figure 5.3 shows that under HVE there was a major spot with a relative mobility (R.M.) compared to  $\text{P}_i$  of 0.50. Two minor spots were also apparent on the chromatogram (R.M.'s 1.00 and 0.64). Whilst the uppermost spot corresponded to the position of free phosphate, the other small



**Figure 5.2** TLC of preparative silica column fractions. The fractions were obtained from separation of a triethyl citrate phosphorylation mixture. The separation medium was silica and the solvent system used was  $\text{CHCl}_3/\text{MeOH}$  (80:20 v/v). ■ - very intense ▨ - mildly intense □ - weak staining





**Figure 5.3** High voltage paper electrophoresis of phosphate (Pi), phosphocitrate (PC), and phosphotriethyl citrate (PTEC). The relative mobilities compared to phosphate are 0.78 for PC and 0.50 for PTEC. ■ - very intense ▨ - mildly intense □ - weak staining.

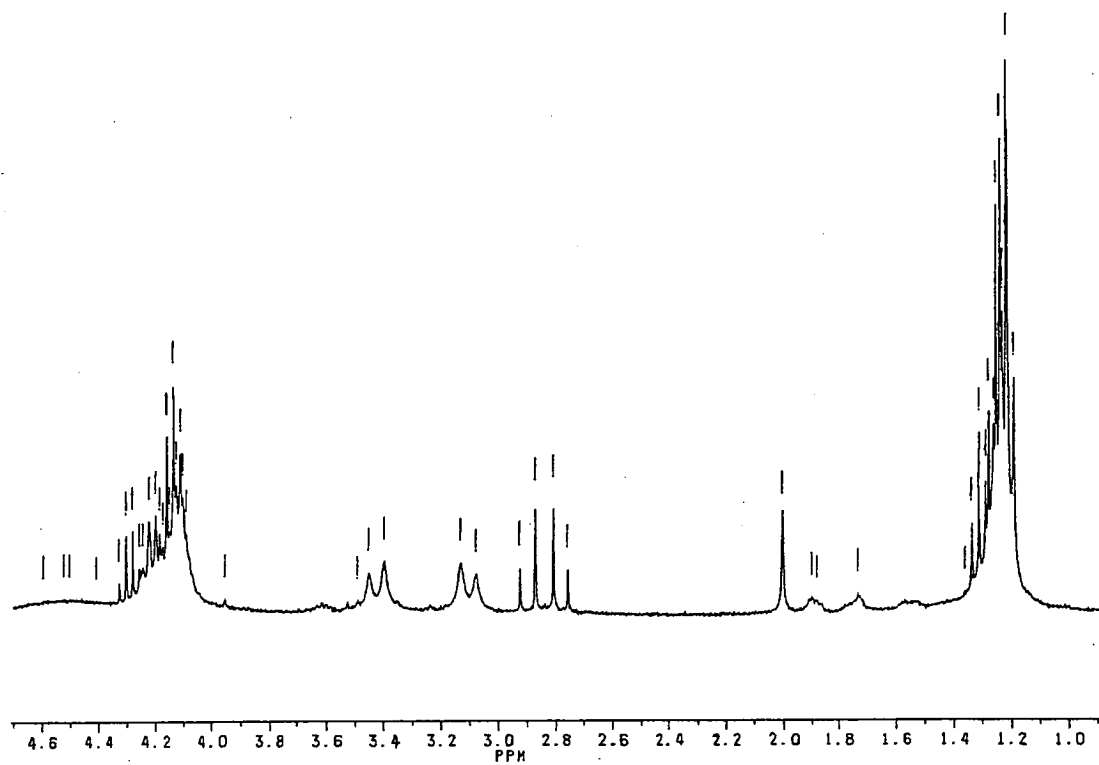
spot was unidentified. PC and inorganic phosphate were included on the chromatogram for reference.

The  $^1\text{H}$ -NMR spectra is presented in Figure 5.4 and the data indicated that PTEC was successfully synthesised but that TEC was present as a contaminant in the preparation. The characterisation of PTEC was supported by analysis for free and hydrolysable phosphate in the mixture. Whilst the presence of free phosphate was apparent from examination of electrophoretograms, analysis showed that Pi contamination was not significant ( $<1\%$ ). Analysis of hydrolysable phosphate indicated that the purest preparations contained PTEC in 90-100% of theoretical yield.

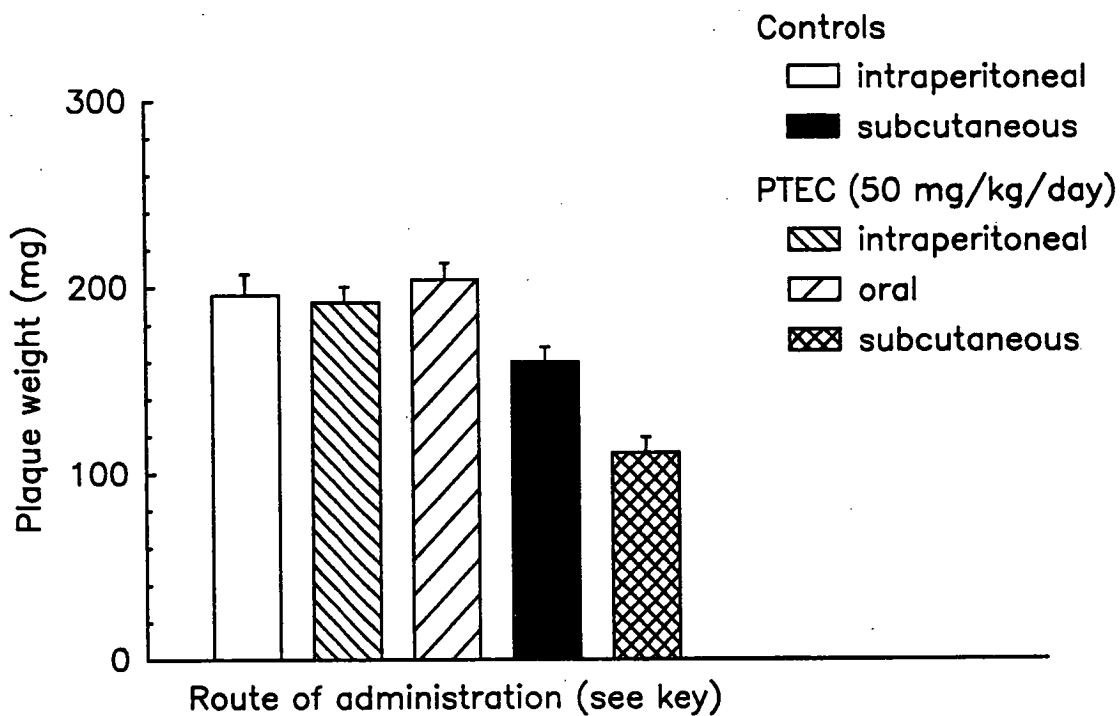
Gas chromatography was also used in the characterisation of PTEC. However, the results from GC were not conclusive as separation could not be achieved under any of the conditions used. Although PTEC eluted in the same position as triethyl citrate, no other peaks were detected. One possible explanation for these results is that PTEC is vulnerable to thermal breakdown yielding TEC and phosphate.

### 5.3.2 Effect of PTEC on calcergy

Figure 5.5 shows the effect of PTEC on plaque calcification. The administration of PTEC by the intraperitoneal and oral routes had no significant effect on plaque formation (intraperitoneal,  $0.40 > p > 0.30$ ; oral,  $p > 0.40$ ). However, when administered subcutaneously at the calcergic site PTEC reduced plaque formation by 31% ( $p < 0.0005$ ) compared to the subcutaneous injection of isotonic saline. Of interest, the administration of saline subcutaneously also reduced plaque formation (15%,  $0.01 > p > 0.0005$ ) when compared to oral and intraperitoneal controls. The mechanism of this



**Figure 5.4**  $^1\text{H}$ -NMR spectra of PTEC



**Figure 5.5** Effect of PTEC on calcergic plaque formation. Data represents mean  $\pm$  SEM. (n = 12-16 plaques).

action is unknown but it is clear that there was no difference between control rats which had been dosed with saline via the oral and intraperitoneal routes.

The minimal inhibition seen when PTEC was administered subcutaneously may have been the result of enzymatic cleavage at the site of calcergic plaque formation. Although unlikely, an alternate explanation might be that trace amounts of PC in the PTEC preparation were responsible for the observed reduction in calcergy formation. The presence of PC could not be detected by HVE (Figure 5.3).

Following the failure of PTEC to act *in vivo*, investigations which subjected PTEC to carboxylesterase action *in vitro* were initiated. The data is not shown here because the results proved entirely negative. Ethanol production was not observed following incubation of PTEC with carboxylesterase, indicating that ester hydrolysis had not occurred under the conditions used.

### **5.3.3 Investigation of phosphotrioctyl citrate synthesis**

Characterisation of trioctyl citrate revealed >99% purity by gas chromatography. Mass spectrometry (Figure 5.6) was consistent with trioctyl citrate and TLC showed a single spot.

Following phosphorylation of trioctyl citrate, TLC showed a similar pattern to that usually obtained with the phosphorylation of triethyl citrate (section 2.2.2). Chromatographic separation of phosphotrioctylcitrate from the hydrogenated mixture by silica chromatography was explored without success. Further work was not pursued due to limited time and the indications that short

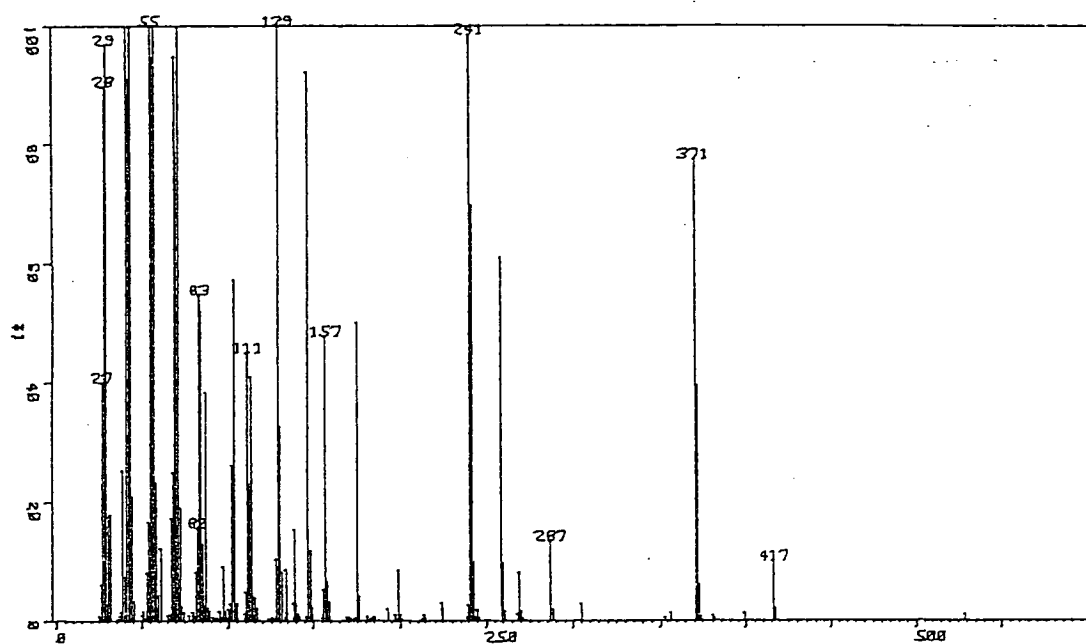


Figure 5.6 Mass spectroscopic analysis of trioctyl citrate

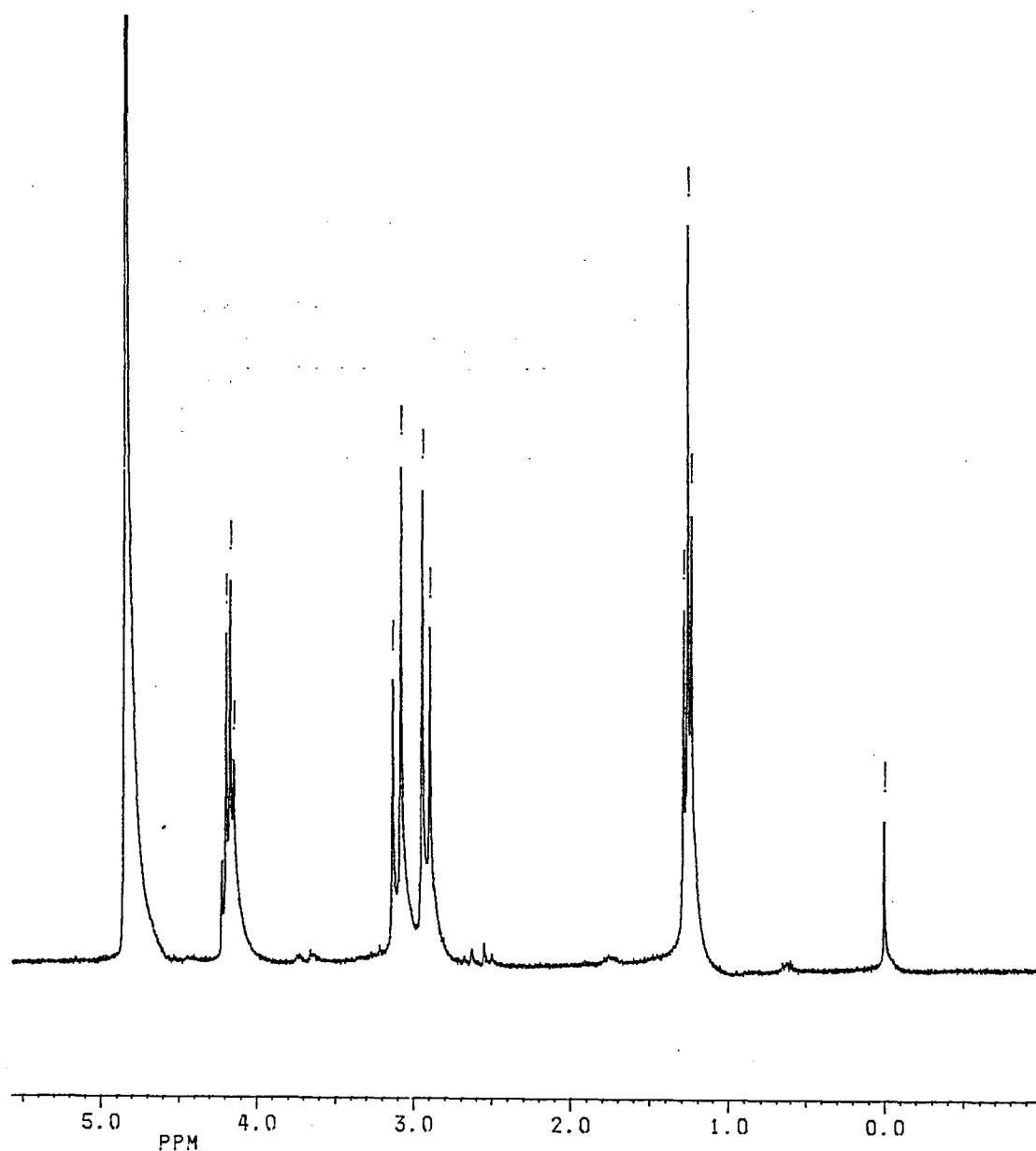
to medium chain alkyl esters of the type envisaged would not eventually lead to active prodrugs of PC.

#### 5.3.4 Monoesters of PC

The inability of PTEC administration to result in inhibition of calcified plaque formation led to the notion that monoesters of PC might have prodrug potential. Malhotra *et al.* (1978) has reported that the specificity of carboxylesterases can totally prevent hydrolysis of some ester compounds. Hence consideration was given to the possibility that *in vivo* hydrolysis of the  $\beta$ -ester linkage on PTEC might have been a limiting factor. Additionally, Lehninger (1983) has reported that the final ester linkage on PTEC is difficult to remove during hydrolysis and Tew *et al.* (1980) have reported that a monoethyl ester of PC did not inhibit the *in vitro* growth of hydroxyapatite crystals.

Monoester synthesis was found to be possible using the controlled hydrolysis conditions described by Meyer *et al.* (1959).  $^1\text{H}$ -NMR (Figure 5.7) of the ethyl monoester prepared by this method indicated that it was the  $\beta$ -ester linkage which remained intact. This was apparent because the splitting pattern which is characteristic of the symmetrical  $\text{CH}_2$  groupings in PC was present. Esterification in the  $\alpha$ -position would have altered the symmetry of the molecule.

Attention was then turned to the direct esterification of PC. Given that there are problems associated with undesirable hydrolysis of PC under conditions combining high temperatures and acidity, a mild esterification method was sought. Blossey *et al.* (1973) have reported the use of polymer linked  $\text{AlCl}_3$  as a mild catalyst for esterification. This strategy was tested and



**Figure 5.7**  $^1\text{H}$ -NMR spectra of mono-ethyl phosphocitrate



analysis of monoester mixtures from syntheses using this reagent revealed the presence of free phosphate, indicating that here again some unwanted hydrolysis of PC had occurred. Preparations when analysed using HVE showed that high concentrations of PC still remained and was obviously not converted to its monoester form by the technique.

The strategies devised for monoester synthesis highlighted some of the problems associated with synthetic work involving PC. One problem which proved insoluble was that the polymer linked catalyst needed to be activated by solvents such as, benzene or CS<sub>2</sub>, which are capable of swelling the polymer. However an aqueous component could not be avoided in the mixture as PC is not soluble in bead activating organic solvents. Problems associated with limited solubility or insolubility in most organic solvents and related to unwanted hydrolysis from combinations of increased temperature and acidity suggested the need for more novel ways of producing monoesters of PC if the prodrug avenue was to be pursued. The monoesters prepared in this study were not trialled using the calcergy model as they were considered to have no greater biological potential than the PTEC preparation.

### 5.3.5 General comments

The synthesis of PTEC has been reported previously (Marcot *et al.*, 1986) and the authors claimed that administration of the compound to rats resulted in dramatic inhibition of urinary stone formation when PTEC was administered at a dose level of 2 µg/day. This seems highly unlikely as the PC dose needed to achieve 50 % inhibition of calcergic plaque formation was found to be 20 mg/kg/day. Further, Sallis *et al.* (1988) using an infection stone model in rats, observed that PC given at 25 mg/kg/day did not elicit a statistically significant reduction in bladder stone formation. Assuming that Marcot *et al.*

(1986) used rats weighing approximately 200 g, a factor of at least 2000 difference in the doses required for an effect is apparent. Whilst PTEC, if active as a prodrug of PC, might be expected to have increased activity if the plasma half life was extended, a factor of at least 2000 seems totally unrealistic.

During these studies chromatographic separation on either silica or alumina did not result in complete isolation of PTEC. However following the failure of the compound to act in the prevention of calcinosis, further work on its separation was considered unnecessary.

## CHAPTER 6

### GENERAL DISCUSSION AND CONCLUSION

During the last decade, knowledge gained with animal models concerning the ability of naturally occurring PC to ameliorate the damaging effects of several disease states has been steadily accumulated (Williams and Sallis, 1981; Shankar *et al.*, 1986; Tsao *et al.*, 1988; Sallis *et al.*, 1988; Cheung *et al.*, 1990). Perhaps the most important and extensive data to date concerning the inhibitory power of PC are that which implicates the compound as having a potential role in preventing the progression of atherosclerosis. This role, first suggested by Williams (1981) one of the early workers in the PC field, if eventually expanded clinically may have considerable benefit. Unless atherogenesis can be controlled serious medical consequences such as myocardial infarction, stroke, gangrene and aneurysm can result (McGill, 1988). This has an enormous economic and social burden on the community and the cost of this disease to the economy of the United States of America has been estimated at 60 billion dollars annually (Comai *et al.*, 1985).

Another major area where it may be possible to utilise the potential of PC is in urolithiasis. Lithotripsy, a non invasive technique to remove stones, once thought to be a panacea, is no longer regarded with such reverence, as severe oedema and destruction of kidney tissue can result from the treatment (Karlsen *et al.*, 1991). Further, lithotripsy does not address the problem of recurring stone formation and so chemical treatment via an inhibitor is an excellent option post-surgically. For urinary tract stone formation PC has

already proven useful in preventing the formation of mixed stones in rats (Sallis *et al.*, 1988).

To date, studies testing the efficacy of PC have administered the compound by intraperitoneal injection. Two aspects of this historical approach needed to be addressed. Firstly, information concerning the most effective way of delivering PC such that its potential could be maximised needed to be gained, and secondly, given that oral delivery of drugs is usually preferred in clinical situations, the characteristics of PC absorption from the intestine needed to be delineated. In this light the studies presented here were undertaken. It was hoped that transport parameters for PC could be delineated, and hence provide an invaluable guide to the direction that future biochemical and pharmaceutical studies should take.

The first area to be studied was the determination of the anticalcifying ability of PC when administered by different routes. The animal model selected was subcutaneous calcergy, described by Doyle *et al.* (1979) which previously had been found suitable for the testing of anticalcifying agents. Additional qualification for the model was that subcutaneous calcergy allowed comparison of the oral and intraperitoneal administration routes as PC must first enter the systemic circulation before passing to the site of calcification. In contrast, the dystrophic transplant model which utilised intraperitoneal implants and was used by Shankar (1986) would have been unsuitable as in that system the calcification target was within the intraperitoneal cavity. Other advantages of the calcergy model included the ease with which it could be performed. Apart from the initial injection of  $\text{KMnO}_4$ , the rats were subjected to minimal trauma for the duration of the experiment and complex dietary and/or surgical techniques were not required.

A detailed dose response curve was constructed to create a model of PC effect on calcergy. The calcergic plaques were analysed by weight, calcium and phosphate content. The analysis according to weight indicated a non-linear reduction in calcergic plaque formation when PC was given by the intraperitoneal route. This was mirrored by the reduction in calcium and phosphorus content of plaques. The inhibition of calcergic plaques by PC was shown to be route dependant and a comparison of the responses to oral and intraperitoneal administration indicated that PC was 45 times less effective when administered by gavage.

When PC was administered subcutaneously at a dose level of 50 mg/kg/day complete inhibition of plaque formation was observed. If future studies regarding the ability of subcutaneously administered PC to prevent calcergy indicate that low doses of this naturally occurring compound are effective, then the possibility of using PC to prevent subcutaneous calcification in humans could be considered. Such disorders include calcinosis universalis and myositis ossificans progressiva. The chance of success with PC would appear high as orally delivered EHDP has been used with beneficial results in the treatment of these two conditions (Cram *et al.*, 1971). Not all types of calcinosis known might be relieved as there is evidence that EHDP is less useful against calcinosis induced by dermatomyositis and scleroderma (Metzger *et al.*, 1974). If a slow release system for PC is a realistic future development, then the potential for using the compound in the treatment of pathological calcinosis may be further enhanced.

The reduced bioavailability following oral administration implied by the calcergy studies was explored using several techniques which allowed absorption from the intestine to be studied more directly. *In situ* loop experiments revealed

a rapid disappearance of PC from the intestinal lumen but the inference of complete uptake of PC was not supported by the everted sac experiments. At low concentrations of the compound in incubation fluids, no transfer of PC was observed. At higher concentrations only limited transfer was detected. However, the non physiological conditions of the *in vitro* studies may have obscured PC transport and this idea has been discussed in chapter 3. Perfusion experiments *in vivo*, confirmed that PC was absorbed from the intestine as the compound was found in portal vein samples. Again, the studies emphasised the limitations of the transport process.

In terms of a transport mechanism for PC, unlike absorption from renal tissue (Wright, 1985), the absorption of carboxylic acids from intestinal tissue has been poorly studied in the past. A tricarboxylate carrier has been identified in liver mitochondria (Claeys and Azzi, 1989) and renal brush borders (Wright *et al.*, 1980) but a similar transporter has not been isolated in intestinal tissue although Browne *et al.* (1978) have demonstrated empirically that tricarboxylic acids are transported by an active mechanism. Wolfram *et al.* (1990) have further characterised tricarboxylic acid transport through work with intestinal brush border membranes of calves. In respect to PC, the compound might be capable of undergoing passive transport. However it is generally acknowledged that highly charged species have difficulty in crossing biological membranes (Schanker, 1971). In relation to the intestine it would be expected that the characteristics of the epithelial barrier might result in limited absorption of PC.

Additional to the transport observations, some evidence of biotransformation was obtained. This was seen in the appearance of radioactive citrate in the portal vein following perfusion of the intestine with 1,5-<sup>14</sup>C-PC and supported by data obtained from the everted sac experiments whereby

some 25% of the dose initially present was found as citrate after 1 h. However, a number of factors were suggestive that hydrolysis of PC to citrate was limited. Firstly, a lag time between the beginning of a perfusion and the appearance of PC in the portal vein was observed and secondly, citrate transfer via an active mechanism (Browne *et al.*, 1978; Wolffram *et al.*, 1990) is probably more rapid than PC transfer. Together these factors indicate that it would be false to base an estimation of the extent of metabolism on a comparison of the proportions of PC and citrate detected in the portal vein. In support of this it was found that, unlike the significant increases in citrate concentration noted in the mucosal fluid during everted sac experiments, citrate did not accumulate extensively in the intestinal lumen during perfusions. Additionally, the return of radioactive metabolites from the systemic circulation to the portal circulation could be another complication. Certainly the *in vitro* metabolism observed appeared to be rate limited. Whilst the extent of this metabolism would need to be further quantified using more extensive experiments that can be better performed following the development of an ultrasensitive assay for PC, these studies did not indicate that metabolism of PC was a limiting factor in absorption.

Further experiments which measured the intravenous disappearance of PC provided valuable insights into the pharmacological profile of PC. Indications of a short half-life for PC in blood combined with poor absorption across biological membranes would be additive, resulting in the poor response of calcergic plaque reduction to orally administered PC. Jung *et al.* (1970) measured the disappearance of pyrophosphate from plasma following intravenous administration in dogs and found that 90% disappeared within 10 min of giving the intravenous dose. Whilst comparison with the detailed kinetic experiments carried out by Jung *et al.* (1970) is not appropriate, the disappearance data for PC indicated a very rapid removal (90% in 1 min). The

role of compartmental metabolism and uptake was not explored in these studies but it is noted from the studies of Williams (1981) that bone had a high affinity for PC (40% uptake of radioactivity within 30 min of an I.V. dose) and that general tissue uptake would certainly explain the high rate of PC disappearance.

Regarding the absorption studies, the gastrointestinal transport of PC was confirmed using several methodologies and as suggested by the hydrophilic nature of PC this transport appeared limited. Hence the need for a mechanism to improve the absorption of PC was apparent. One logical rationale for increasing absorption was to develop a lipophilic prodrug of PC. This seemed appropriate in the light of success obtained with other compounds. Two notable examples of derivatisation being used to improve bioavailability of the parent compound are prodrugs of ampicillin and mecillinam (Bundgaard and Hansen, 1981).

In this regard the synthesis of simple alkyl esters of PC was examined. The triethyl ester of PC proved ineffective in preventing plaque formation when tested using the calcergy model. Several reasons for this could be postulated. Whilst it was expected that the triester might be well absorbed, confirmation of this would require detailed radiolabelling experiments. A slow rate of hydrolysis in blood and tissues is another consideration which could have resulted in insufficient PC becoming available for an effective response. It is possible that only partial hydrolysis occurred which would result in remaining ester linkages preventing the compound from having any useful anticalcifying ability (Tew *et al.*, 1980). The positioning of an ester moiety would be critical as it can be postulated that a  $\beta$ -monoester, as compared to an  $\alpha$ -monoester, would be generally less suitable as a prodrug due to a slower hydrolysis rate (Meyer *et al.*, 1959).



Some thought has to be given to the design of future PC prodrugs especially in regard to the nature of the epithelial barrier and hydrolytic enzymes available for bioconversion. The alkyl chain length of the ester is known to influence the release of the parent molecule from the prodrug. Kawaguchi *et al.* (1990) found that hydrolysis of ester prodrugs of zidovudine was highly dependant on the length of the acyl chains contained within the molecule. The positioning of ester groups can also have relevance regarding the absorption rate. For example, Jandacek *et al.* (1987) found that triglycerides were more efficiently absorbed when they contained octyl groups in the 1 and 3 positions and a long chain fatty acid in the 2 position, as opposed to more typical long chain fatty acid triglycerides.

It is important to note that a monoester of PC will have reduced lipophilicity compared to its di- or triester and this compromise between charge and lipophilicity will effect the rate of absorption. Regarding this issue, White *et al.* (1980) found that mono or diesterification of dicarboxylic acids was an important factor in predicting absorption of drugs. In their example, phthalate monoesters were absorbed preferentially to the corresponding diesters. The authors claimed that the unstirred water layer probably played an important role as the ionic charge on the monoester ( $pK_a=3.0$ ) would allow them to be available for absorption preferentially to the diesters. Consequently, the ideal prodrug form of PC may be a monoester esterified on an outer carboxyl group with an easily hydrolysable side chain. Problems concerning the eventual hydrolysis of ester linkages within PC prodrugs would need to be fully explored *in vitro* to ascertain that future compounds contain bioreversible linkages.

The ester compounds discussed so far relate to aliphatic derivatisations. One of the keys to achieving a useful prodrug configuration of PC may be the

use of a specific ester form which can be more easily hydrolysed *in vivo*. The prodrug itself may ideally have a short biological half-life and be subject to enzyme action in blood or certain tissues. Nielsen and Bundgaard (1988) have used glycolamide esters as prodrugs of carboxylic acids. These compounds have short half-lives in blood and are thought to be hydrolysed by cholinesterases. Hence if a glycolamide monoester of PC can be developed, this prodrug may prove to be a highly effective oral delivery system for PC.

An additional useful strategy in the development of PC prodrugs might be site specific delivery of PC, in a prodrug form, to a relevant tissue (eg. kidney) for conversion to active PC. An example of this concept is the use of gamma-glutamyl prodrugs which are directed to the kidney and hydrolysed by gamma-glutamyltranspeptidase (Stella and Himmelstein, 1980). Another possible tool might be the development of lipid drug delivery systems based on fat pellets, solid lipid dispersions, microemulsions or liposomes (Eldem and Speiser, 1989). Kamperman (1989) has explored this area in regard to increasing the biological half-life of PC following parenteral administration with some success. However, limited entrapment due to the hydrophilic nature of PC, in the carriers trialled has so far proved a restriction. The potential of lipid systems would be enhanced if a suitable lipophilic form of PC was available synthetically for combining in a lipid vehicle.

In conclusion, PC was found to have limited absorption from the intestine, a factor which severely diminished its efficacy. The studies suggest that further work relating to the biological half-life, tissue distribution, and metabolism of PC would be beneficial. However the development of a form of PC which is more extensively orally absorbed would facilitate the presentation

of PC as a drug compound. Given the exciting anticalcifying potential of PC this task would seem a priority.

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